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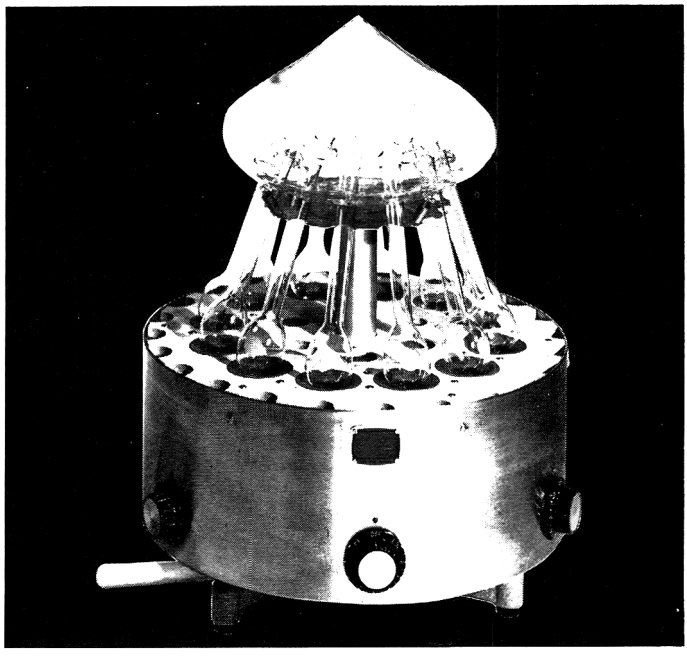

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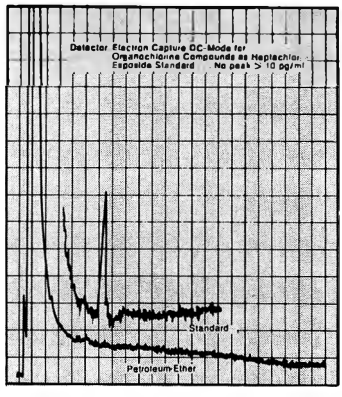

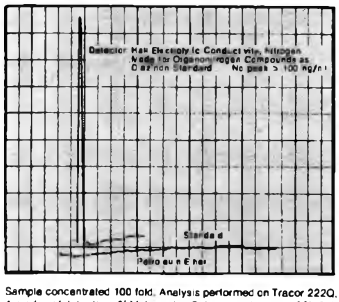

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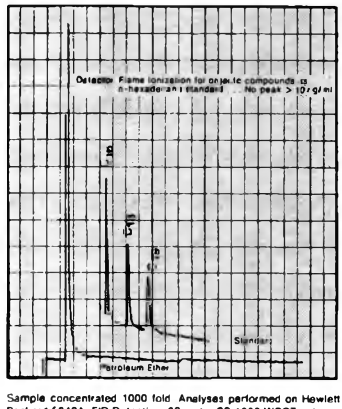

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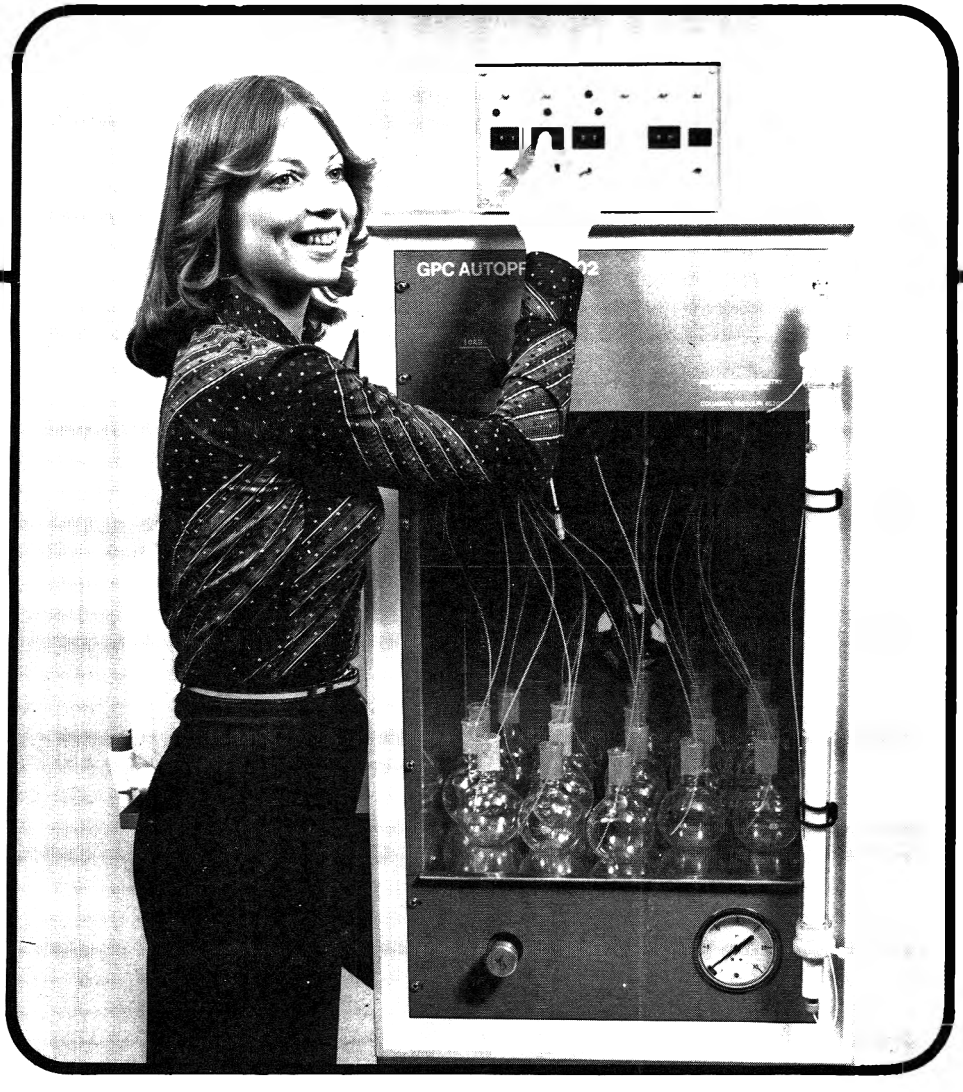

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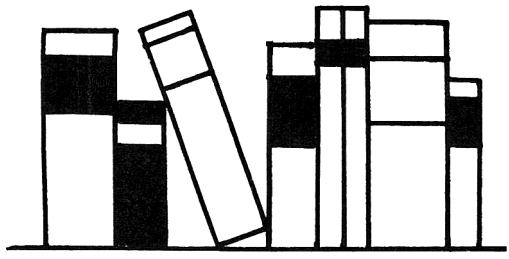

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Related to Agriculture and the Public Health

Vol. 65 JANUARY 1982 No. 1

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Symbols and Abbreviations

kg	kilogram(s)
g	gram(s)
mg	milligram(s)
μg	microgram(s)
ng	nanogram(s)
L	liter(s)
mL	milliliter(s)
μL	microliter(s)
m	meter(s)
cm	centimeter(s)
mm	millimeter(s)
μm	micrometer(s) (*not* micron)
nm	nanometer(s) (*not* millimicron)
A	ampere(s)
V	volt(s)
dc	direct current
ft	foot (feet)
in.	inch(es)
cu. in.	cubic inch(es)
gal.	gallon(s)
lb	pound(s)
oz	ounce(s)
ppm	parts per million
ppb	parts per billion
psi	pounds per square inch
sp gr	specific gravity
bp	boiling point
mp	melting point
id	inside diameter
od	outside diameter
h	hour(s)
min	minute(s)
s	second(s)
%	percent
⅌	standard taper
N	normal
M	molar
mM	millimolar

(Note: Spectrophotometric nomenclature should follow the rules contained in *Official Methods of Analysis*, "Definitions of Terms and Explanatory Notes.")

MYCOTOXINS

High Performance Liquid Chromatographic Determination and Clearance Time of Aflatoxin Residues in Swine Tissues

DORIS M. MILLER, DAVID M. WILSON,[1] ROGER D. WYATT,[2]
JERRY K. McKINNEY,[1] WAYNE A. CROWELL, and BARRY P. STUART
University of Georgia, College of Veterinary Medicine, Department of Veterinary Pathology, Athens, GA 30602

Liver, kidney, and muscle tissues from swine given a single oral dose of 1.2 mg aflatoxins/kg body weight and from young pigs fed an aflatoxin-contaminated diet (0, 400, or 800 ng/g feed) for 10 weeks were analyzed for aflatoxin residues. Extraction was performed according to the method of Stubblefield and Shotwell. Aflatoxin residues were detected by high performance liquid chromatography (HPLC), and residue levels were correlated with withdrawal times. Residues were present in liver and kidney but not muscle by 12 h post-dosing; residues were detected in muscle after 24 h. Residue levels decreased over the 72 h period in all tissues. No aflatoxin residues were detected in the muscle from any pig 72 h post-dosing or in those pigs fed the 400 ng/g diet, but were present in the muscle of pigs fed the 800 ng/g diet.

Aflatoxins are probably the most thoroughly investigated mycotoxins. The presence of aflatoxins in animal feeds is a health hazard to animals and a potential public health hazard (1) because exposed animals can retain residues of aflatoxins or their metabolites in their tissues (2–4). There may be a direct relationship between human consumption of contaminated foodstuffs and cancer because liver tumors occur at a high rate in countries where consumption of aflatoxin-contaminated foods is commonplace (5).

Two principal detection methods, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) (6), are used to assay aflatoxins. Recent investigations have shown that HPLC is a useful technique for determining aflatoxins in feeds and foods, with excellent resolution of the individual aflatoxins (6–8). Normal phase HPLC analysis of tissues from pigs experimentally fed known amounts of aflatoxin has not been previously reported; however, Gregory and Manley (9) recently described reverse phase HPLC method for detecting aflatoxins in animal tissues and products. They used TFA derivatives of B_1, G_1, and M_1 in their method and obtained good resolution. Aflatoxin B_{2a} can occur naturally and this method would add the B_1 derivative to the B_{2a} present in the tissue. Results obtained by using normal phase and reverse phase HPLC should not be different. The use of a packed cell gives about the same sensitivity for normal phase HPLC as TFA derivative with fluorescence detection in reverse phase HPLC. With continued research and simplification of extraction methods, HPLC will improve the accuracy and precision of aflatoxin measurement in numerous agricultural products and animal tissues.

The present investigation reports on levels of aflatoxin residues extracted from tissues of swine with known aflatoxin withdrawal times and detected by normal phase HPLC.

METHOD

Protocol

In a chronic study, 30 mixed breed feeder pigs (10 animals/diet) were fed a diet prepared from naturally contaminated corn containing either 0, 400, or 800 ng total ($B_1 + B_2 + G_1 + G_2$) aflatoxins/g feed. Concentrations of individual aflatoxins are shown in Table 1. The pigs were fed the diets continuously without a withdrawal period for 10 weeks before euthanasia.

In the acute study, 8 feeder pigs were dosed with a single oral dose of a rice powder–water slurry containing 1.2 mg total aflatoxins ($B_1 + B_2 + G_1 + G_2$)/kg body weight. Eight additional pigs served as controls. One treated pig died at

[1] University of Georgia, Department of Plant Pathology, Tifton, GA 31793.
[2] University of Georgia, Department of Poultry Science, Athens, GA 30602.

004-5756/82/6501-0001–04$01.00

Table 1. Equivalent amounts (ng/g) of individual aflatoxins in diets and rice powder

Dosage	B_1	B_2	G_1	G_2
1.2 mg total aflatoxins/kg body weight[a]	972	Tr[b]	228	Tr
400 ng total aflatoxins/g diet[c]	300	56	40	4
800 ng total aflatoxins/g diet[c]	600	112	80	8

[a] Administered in single oral dose of rice powder–water slurry.
[b] Tr = trace amounts found.
[c] Diet prepared from naturally contaminated corn. Values shown are means of 10 determinations, thin layer chromatography.

12 h post-dosing. Two treated and 2 control pigs were euthanized at 24, 48, and 72 h. Histopathologic, electron microscopic, and clinical pathologic results observed within these pigs are reported in separate publications.

The feed and rice powder inoculum were analyzed for aflatoxin content by the Pons rapid cottonseed method, **26.A09-26.A16** (10).

Apparatus and Reagents

(a) *High performance liquid chromatograph.*—Waters (Waters Associates, Inc., Milford, MA 01757) 6000A pump and U6K injector; Zorbak® Sil 4.6 × 25 cm column (DuPont); Fluorichrom™ (Varian) fluorescence detector equipped with silica gel-packed cell, 365 nm excitation (7-54 and 7-60, Corning glass color designation) and 440 I emission filters; and Model No. 9176 Varian dual pen recorder.

(b) *HPLC mobile phase.*—3.5 L: 60% water-saturated $CHCl_3$ (1544 mL water-saturated $CHCl_3$ + 1029 mL $CHCl_3$)–cyclohexane (700 ml)–acetonitrile (140 mL)–ethanol (77.3 mL).

(c) *Aflatoxin standards.*—Applied Science Laboratories, Inc., State College, PA 16801. Prepare using HPLC mobile phase as diluent as described in ref. 7 to contain 0.5 μg aflatoxins B_1 and G_1 and 0.15 μg aflatoxins B_2 and G_2/mL. Prepare separate M_1 standard to contain 1 μg aflatoxin M_1/mL.

Extraction

Use method of Stubblefield and Shotwell (11). Weigh 100 g tissue and blend into paste. Transfer paste to flask and add 10 mL 20% citric acid solution, 20 g diatomaceous earth, and 200 mL methylene chloride. Shake 30 min, and then filter mixture through paper into Erlenmeyer flask containing 15 g Na_2SO_4. Mix filtrate 2 min, and then refilter. Record volume of filtrate and evaporate to near dryness on steam bath.

Column Cleanup

Dissolve extract in 15 mL hexane-$CHCl_3$ (1 + 1), and transfer to column prepared according to method of Stubblefield and Shotwell (11). Rinse beaker and column with additional portions of hexane-$CHCl_3$ (1 + 1) (4 mL total). Sequentially elute column with 25 mL glacial acetic acid–toluene (1 + 9), 25 mL hexane, 25 mL acetonitrile–ether–hexane (1 + 3 + 6), and 40 mL acetone-$CHCl_3$ (1 + 4). Collect acetone-$CHCl_3$ fraction and evaporate to near dryness on steam bath, transfer residue quantitatively to small vial with $CHCl_3$, and evaporate under vacuum to dryness.

High Performance Liquid Chromatography

Stabilize HPLC flow rate at 1.3 mL/min giving pump pressure of ca 1000 psi, chart speed of 1 cm/min, detector on low gain, low lamp, with attenuation of 10. Separation of B_1, B_2, G_1, and G_2 is described by Thean et al. (12). M_1 has retention time of ca 13 min. Typical chromatograms of spiked tissue show baseline resolution of all 5 aflatoxins with no interfering peaks. Retention times are reproducible to ±1% or better.

Dissolve dry sample extract with 100 μL mobile phase. Inject 10 μL sample extract and record chromatograph for 30 min to allow M_1 elution. Record peak heights and retention times of known quantities of aflatoxin standards (B_1, B_2, G_1, G_2, and M_1) daily for quantitation. Note and measure peak heights (mm) and retention times for each possible aflatoxin peak in samples. Calculate aflatoxin levels as described by Stubblefield and Shotwell (11), using peak heights for sample and standard peaks.

Confirmation

Confirm by using trifluoroacetate (TFA)-treated thin layer chromatograms of aflatoxins B_1, G_1, and M_1, according to the method of Trucksess and Stoloff (4).

Results and Discussion

Several experiments have been conducted to determine tissue residue levels in swine after either acute or chronic intoxication (2–4).

Table 2. Aflatoxin residues in swine tissues (ng/g, wet matter basis) [a] from 30 swine fed naturally contaminated diets for 10 weeks with no withdrawal time

Aflatoxins, ng/g diet	Liver					Kidney					Muscle				
	B_1	B_2	G_1	G_2	M_1	B_1	B_2	G_1	G_2	M_1	B_1	B_2	G_1	G_2	M_1
800	1.57	0.17	ND[b]	ND	1.07	0.25	0.05	ND	ND	0.91	0.19	ND	ND	ND	0.45
400	0.51	0.03	0.31	ND	0.58	0.20	0.02	ND	ND	0.61	ND	ND	ND	ND	ND
0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Each value represents the mean for 10 animals.
[b] Not detected.

However, most of these experiments specified TLC methods with or without densitometry as the method of aflatoxin quantitation and were designed only to show the presence of tissue residues without regard to clearance times. The present study specifies an extraction technique devised by Stubblefield and Shotwell (11), with normal phase HPLC as the primary method of detection. Recovery experiments carried out with this method showed recoveries of M_1 and B_1 from 85 to 115% at levels of 0.1–5 ng/g, with at least 3 determinations on samples spiked at 0.1, 0.5, 2.5, and 5 ng aflatoxins B_1 and M_1/g.

The levels of aflatoxins B_1, B_2, G_1, G_2, and M_1 detected in swine tissues by using HPLC are summarized in Tables 2 and 3. The equivalent amounts of individual aflatoxins in the feed are presented in Table 1. The presence of possible M_2 and other aflatoxin metabolites was observed in some tissues but these were not quantitated because of lack of available standards.

In our study on tissue levels of aflatoxins B_1, B_2, G_1, G_2, and M_1 in swine fed diets naturally contaminated with aflatoxin or dosed with an aflatoxin–rice powder slurry, both B_1 and M_1 were present in the muscle of pigs fed the 800 ng/g diet (with no withdrawal time). Table 1 shows levels of individual aflatoxins in both diet and rice powder. No detectable residues were found in muscle from swine fed the 400 ng/g diet (with no withdrawal time) or at 72 h post-dosing in the 1.2 mg/kg body weight exposure. Aflatoxins were not detected in the muscle of a pig receiving 1.2 mg aflatoxin/kg and dying 12 h after dosing. Residue levels were consistently lower at 72 h than at 24 h post-dosing for pigs administered 1.2 mg aflatoxin/kg body weight. Aflatoxins were not detected in the control tissues.

The rates of clearance of the different aflatoxins (B_1 vs G_1) depend on their different chemical structures (13), and on route of exposure, dose, diet, sex, and species (14). Addition of the second lactone ring to the B_1 molecule to form G_1 reduces the activity and clearance of G_1 vs B_1. The greater polarity of the M_1 molecule makes it more water-soluble, facilitating rapid removal once formed. In the present experiment, the diet, sex, dose, and species were all the same, thus eliminating these factors as variables in the clearance of the aflatoxins.

The various aflatoxins and their metabolites

Table 3. Aflatoxin residues in swine tissues (ng/g, wet matter basis) [a] after a single LD_{50} dosage (1.2 mg aflatoxins/kg body weight) [b]

Hours post-dosage	Liver					Kidney					Muscle				
	B_1	B_2	G_1	G_2	M_1	B_1	B_2	G_1	G_2	M_1	B_1	B_2	G_1	G_2	M_1
12	9.00	0.64	0.47	0.08	5.44	3.80	0.29	0.60	0.07	3.30	ND[c]	ND	ND	ND	ND
24	6.65	0.17	0.11	0.03	16.80	1.50	1.52	0.45	ND	2.10	1.50	0.28	ND	ND	2.18
	2.53	0.37	0.53	0.03	5.17	0.64	0.08	ND	ND	4.10	0.47	0.03	ND	ND	0.68
48	3.31	0.75	0.25	ND	1.70	0.47	0.24	ND	ND	1.11	1.57	0.45	ND	ND	0.66
	1.22	0.13	0.09	ND	1.29	1.92	0.23	0.19	ND	4.00	0.70	0.07	ND	ND	0.33
72	0.15	0.04	ND	ND	0.30	0.16	0.03	ND	ND	0.28	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	0.39	0.01	ND	ND	0.01	0.23	0.06	0.08	0.04	0.04	ND	ND	ND	ND	ND

[a] Each set of values represents results for a single animal.
[b] No aflatoxin was detected in any of the tissues from 8 control pigs (2 euthanized at 24 and 48 h, 4 euthanized at 72 h post-dosing).
[c] Not detected.

are carcinogenic in several animal species at low doses (1, 5, 15, 16). Wogan et al. (15) reported hepatomas in rats fed 1 ng/g diet after 104 weeks and Butler et al. (16) reported irreversible cellular hepatocyte changes by 6 weeks in rats on diets containing 5 ppm aflatoxin. A higher rate of liver cancer is present in countries in which the people consume aflatoxin-contaminated diets (5). The levels present in the tissues in this experiment could be considered significant not for their acute toxicity potential but for their carcinogenic potential after chronic consumption by man.

Our residue results are in general agreement with results of others who dosed young swine with high levels of aflatoxins (3, 17), without withdrawal times before analysis. Krogh et al. (2) reported consistently higher aflatoxin residues within the tissues of the pigs in his study compared with our results and others. In a recent experiment, Neff (14) fed various levels of aflatoxin to young swine and reported residue levels similar to ours, with no detectable aflatoxin residues found in tissues 4 days after aflatoxin withdrawal. The swine tissues analyzed by Gregory and Manley (9) were obtained from Neff's experiment. They used reverse phase HPLC and were in agreement with Neff's TLC results. The absence of detectable residues in one of 3 pigs at 72 h and the low level of residues in the other pigs at this time confirms the clearance of detectable aflatoxin residues by 4 days as reported by Neff (14).

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References

(1) Stoloff, L. (1977) in *Mycotoxins in Human and Animal Health*, J. V. Rodricks, C. W. Hesseltine, & M. A. Mehlman (Eds), Pathotox Publishers Inc., Park Forest South, IL, pp. 20–23
(2) Krogh, P., Hald, B., Hasselager, E., Madsen, A., Mortensen, H. P., Larsen, A. E., & Campbell, A. D. (1973) *Pure Appl. Chem.* **35**, 275–281
(3) Furtado, R. M., Pearson, A. M., Hogberg, M. G., & Miller, E. R. (1979) *J. Agric. Food Chem.* **27**, 2351–2354
(4) Trucksess, M. W., & Stoloff, L. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 1080–1082
(5) Newberne, P. M. (1974) *Environ. Health Perspect.* **9**, 1–32
(6) Pons, W. A., Jr (1976) *J. Assoc. Off. Anal. Chem.* **59**, 101–105
(7) Pons, W. A., Jr (1979) *J. Assoc. Off. Anal. Chem.* **62**, 586–594
(8) Colley, P. J., & Neal, G. E. (1979) *Anal. Biochem.* **93**, 409–418
(9) Gregory, J. F., & Manley, D. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 144–151
(10) "Changes in Methods" (1975) *J. Assoc. Off. Anal. Chem.* 58, 394–395
(11) Stubblefield, R. D. & Shotwell, O. L. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 964–968
(12) Thean, J. E., Lorenz, D. R., Wilson, D. M., Rodgers, K. & Gueldner, R. C. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 631–633
(13) Wogan, G. N., Edwards, G. S., & Newberne, P. M. (1971) *Cancer Res.* **31**, 1936–1942
(14) Neff, G. L. (1980) M. Sc. thesis, University of Florida, Gainesville, FL
(15) Wogan, G. N., Paglialunga, S., & Newberne, P. M. (1974) *Food Cosmet. Toxicol.* **12**, 681–685
(16) Butler, W. H., Hempsall, V., & Stewart, M. G. (1981) *J. Pathol.* **133**, 325–340
(17) Jacobson, W. C., Harmeyer, W. C., Jackson, J. E., Armbrecht, B., & Wiseman, H. G. (1978) *Bull. Environ. Contam. Toxicol.* **19**, 156–161

Colorimetric Determination of Patulin Produced by *Penicillium patulum*

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A quantitative method has been developed for the determination of patulin, a mycotoxin produced by *Penicillium patulum*. The fungus was grown separately in well defined liquid minimal medium, and on rice and bread for 15 days. Patulin was extracted with ethyl ether and determined colorimetrically, using phenylhydrazine hydrochloride. Recovery of pure patulin added to bread and rice was also studied. The lower limit of detection was 1 μg/g.

Food and feeds may become contaminated with fungi, mainly of *Aspergillus* and *Penicillium* genera, under poor storage conditions. A number of secondary metabolites of these genera, isolated from cultures and from contaminated feeds, are toxic to humans and animals (1). It has been reported that patulin, produced by the common food contaminant *Penicillium patulum* (2), is a hepatotoxic (3) as well as carcinogenic (4) metabolite. The LD_{50} value of patulin for mice and rats is approximately 0.3–0.7 mg/20 g body weight when injected intravenously (5). Therefore, a method of analysis to determine the presence and the quantity of toxins in food and feeds is needed.

Chemical and biological methods have been reported for determining many fungal toxins, such as aflatoxin B_1 in corn (6), aflatoxins in groundnuts (7), zearalenone in corn (8), citrinin in food materials (9), sterigmatocystin in cereal grains and soybeans (10), and terreic acid in culture filtrate and contaminated bread and rice (11). Some methods are available for determining patulin (12, 13). We have developed a simple colorimetric determination of patulin produced by *P. patulum*, based on the reaction of patulin with phenylhydrazine, which is measured at 540 nm.

METHOD

Apparatus

(a) *Thin layer chromatographic (TLC) apparatus.*—20 × 20 cm glass plates, applicator, and developing tank.

(b) *Photoelectric colorimeter.*—Klett-Summerson.

Reagents and Materials

(a) *Penicillium patulum.*—Obtained from J. J. Vander Watt, South African Medical Research Council, Pretoria, South Africa.

(b) *Synthetic minimal medium.*—Contains the following (g/L): glucose 40.00, sodium nitrate 2.00, potassium dihydrogen phosphate 1.52, potassium chloride 0.52, magnesium sulfate ($MgSO_4 \cdot 7H_2O$) 0.52, and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) 0.01.

(c) *Raw rice.*—Good quality fresh rice (purchased locally).

(d) *Fresh bread.*—Containing 0.2% calcium propionate as preservative (supplied locally).

(e) *Patulin standard solution.*—Dissolve 10 mg patulin (Makor Chemicals Ltd, Jerusalem, Israel) in 50 mL ethanol.

(f) *Developing solvent.*—Use analytical grade solvents. Toluene–ethyl acetate–formic acid (50 + 40 + 10).

(g) *Phenylhydrazine hydrochloride solution.*—Freshly prepared. Dissolve 2 g phenylhydrazine hydrochloride in 100 mL water.

Preparation of Sample

Separately sterilize 1 L minimal medium (adjusted to pH 6.5), 100 g fresh bread, and 100 g raw rice with 30% moisture 15 min at 10 psi and 120°C. Inoculate each with 1 mL spore suspension of *P. patulum* (10^6 spores/mL, counted by hemocytometer) and let grow 15 days at 30 ± 2°C in stationary phase culture.

Extraction

Filter to remove mycelia from minimal medium. Adjust pH to 2.0 with 2N HCl. Extract with three 20 mL portions of ethyl ether. Combine extracts and concentrate to dryness under vacuum in flash evaporator. Add 10 mL ethanol to extract patulin. Repeat extraction procedure with bread and rice samples.

TLC Determination

Apply slurry (0.25 mm thick) of 11 g silica gel G and 20 mL water to 20 × 20 cm glass plate, using applicator. Activate plate 1 h at 120°C, and cool. Spot with known amount (0.05 mL) of extract and 0.02–0.08 mL patulin standard solu-

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Table 1. Patulin production [a] by *Penicillium patulum* on 3 substrates

Trial No.	Substrate: Minimal medium	Bread	Rice
1	10.67	1.08	0.92
2	10.39	1.13	0.88
3	10.72	1.00	0.94
4	10.60	1.23	0.97
5	10.28	1.17	0.91
Av.	10.53	1.12	0.92

[a] Results are mg/L for minimal medium, and mg/100 g for bread and rice.

Table 2. Recovery of patulin added to bread and rice [a]

Added μg/100 g	Recovery, %: Bread	Rice
0	0	0
100	70	85
200	74	90
300	80	91
400	89	97
500	91	98

[a] Averages of 3 determinations.

tion. Develop plate with toluene–ethyl acetate–formic acid (50 + 40 + 10) in sealed tank. Remove plate after 45 min, air-dry, and spray with phenylhydrazine hydrochloride solution. Keep plate 5 min at 100°C. Mark and separately scrape yellow spots of patulin phenylhydrazone. Extract phenylhydrazone from each scraped sample with 5 mL *n*-butyl alcohol, and centrifuge. Read color intensity of supernate immediately at 540 nm in colorimeter.

Recovery Experiments

Add 100, 200, 300, 400, and 500 μg patulin to separate 100 g portions of fresh bread and raw rice, and determine patulin.

Results and Discussion

Absorbance measurements, using phenylhydrazine hydrochloride, obeyed Beer's law in the concentration range 4–20 μg patulin/mL.

Our laboratory reported earlier (14) on the quantitative estimation of patulin by colorimetric methods, using 3-methyl-2-benzthiazolinonehydrazine hydrochloride (MBTH) and 2,4-dinitrophenylhydrazine hydrochloride (DNPH). We studied the use of MBTH and DNPH, as well as phenylhydrazine hydrochloride, in the present method. Phenylhydrazine hydrochloride performed best, forming a color complex of high intensity. Among the 3 patulin hydrazones, patulin phenylhydrazone is the least soluble in water. To overcome this difficulty, we used *n*-butyl alcohol which is the best solvent for extracting patulin hydrazone, and carried out the measurement in an *n*-butyl alcohol medium.

In the previous study (14), after the patulin was scraped from the TLC plate, the color was developed with MBTH and DNPH. In the present method, we sprayed phenylhydrazine hydrochloride directly and scraped off the yellow patulin phenylhydrazone. This is a simple scraping and identification process, compared with the earlier method, which specifies examination under UV light and scraping the dark brown patulin spot. In the latter case, it is difficult to identify the toxin.

Patulin was extracted from cultures with ethyl ether in the present method and the extracts were concentrated to dryness. The residues were then extracted with 10 mL ethanol, and a 0.05 mL aliquot was applied to the TLC plate. In the previous method the ether extract is concentrated to 10 mL, and a 0.05 mL aliquot is applied to the plate. If ethyl ether were used as the solvent, it would be difficult to obtain an exact volume because of its low boiling point. To overcome this problem, we used ethyl alcohol, which has a higher boiling point and is a good solvent for patulin.

Measurements of patulin indicate that the production of the toxin by *P. patulum* on minimal medium was greater than that on rice and bread (Table 1): 10.5 mg/L minimal medium, 1.1 mg/100 g bread, and 0.9 mg/100 g rice. Production of patulin was optimum between 12 and 17 days.

Recoveries of patulin from bread and rice were quantitative and within the limits of experimental error at a level of 500 μg/100 g substrate (Table 2). This method is less sensitive below 100 μg patulin/100 g.

The method can be useful for screening grains before distribution and consumption.

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References

(1) Miller, M. W. (1961) *The Pfizer Handbook of Microbial Metabolites*, McGraw-Hill Book Co., New York, NY

(2) Scott, P. M., Kennedy, B. P. C., & Van Wallbeek, W. (1972) *Experientia* **28,** 1252.
(3) Madiyalakan, R., & Shanmugasundaram, E. R. B. (1978) *Arogya J. Health Sci.* **4,** 101–108
(4) Dickens, F. & Jones, H. E. H. (1961) *Br. J. Cancer* **15,** 85–100
(5) Broom, W. A., Bulbring, E., Chapman, C. J., Hampton, J. W. F., Thomson, A. M., Ungar, J., Wein, R., & Woolfe, G. (1944) *Br. J. Exp. Pathol.* **25,** 195–207
(6) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **26.049–26.051**
(7) Lee, W. V. (1965) *Analyst* **90,** 305–307
(8) Shotwell, O. L., Goulden, M. L., & Bennett, G. A. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 666–670
(9) Damodaran, C., Ramadoss, C. S., & Shanmugasundaram, E. R. B. (1973) *Anal. Biochem.* **52,** 482–489
(10) Shannon, G. M., & Shotwell, O. L. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 963–965
(11) Subramanian, T., Mohan Namasivayam, K., & Shanmugasundaram, E. R. B. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 581–583
(12) Scott, P. M., & Kennedy, B. P. C. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 813–816
(13) Moller, T. E., & Josefsson, E. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1055–1056
(14) Madiyalakan, R. (1977) Ph.D. thesis, University of Madras, Madras, India

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High Pressure Liquid Chromatography of Zearalenone and Zearalenols in Rat Urine and Liver

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A high pressure liquid chromatographic technique with internal standardization has been developed for determining zearalenone and metabolites in rat urine and liver. Following extraction with methylene chloride and solvent partition, samples are cleaned up by applying the extract to a Sephadex LH-20 column and eluting with a mixture of benzene–methanol (85 + 15). Compounds were resolved on 2 Partisil-10 columns (25 cm × 4.6 mm id) in series with a mobile phase of isooctane–chloroform–methanol (35 + 25 + 3), and detected at 280 nm. The internal standard was 6′α-acetoxyzearalane. Limits of detection were about 2.0 ng for zearalenone and 5.0 ng for zearalenols (6′-hydroxyzearalane). Zearalenone and zearalenols were excreted mainly in free form with relatively little glucuronide conjugation. Metabolism of zearalenone to free zearalenol was minor compared with formation of bound forms.

Zearalenone is a uterotropic mycotoxin produced by *Fusarium* fungi and has recently been described as an important mycotoxin in Canadian corn crops (1). The presence of zearalenone in animal feedstuffs can result in reproductive disorders in many classes of livestock, especially swine (2). Some progress has been made in understanding the metabolic interrelationships between nutrients and zearalenone (3); however, analytical limitations have required the use of radioactive tracers (4).

Numerous procedures have been developed to determine zearalenone in cereals, including thin layer chromatography (TLC) (5), gas-liquid chromatography (6), and high pressure liquid chromatography (HPLC) (7). HPLC detection of zearalenone and isomers of the metabolite zearalenol formed by in vitro preparations of rat liver has also been reported (8). Recently, a method for determining zearalenone and α-zearalenol in blood plasma has been described (9). The method in this paper permits determination with internal standardization of residual zearalenone and the isomers of zearalenol in tissues as well as urine.

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METHOD

Apparatus

(a) *Homogenizer.*—Super Dispax Tissumizer (Ultra-Turrax), SDT series motor with SDT 182 EN shaft and generator (Tekmar Co., Cincinnati, OH).

(b) *Centrifuges.*—Sorvall Superspeed RC2-B (Ivan Sorvall, Inc., Norwalk, CT). IEC clinical centrifuge (IEC Equipment Co., Needham Heights, MA).

(c) *Liquid chromatography.*—Waters 6000A solvent delivery system equipped with Model 440 absorbance detector (280 nm), data module, and WISP 710A automatic injector (Waters Scientific Inc., Mississauga, Ontario). Two 25 cm × 4.6 mm id Partisil-10 columns (Bendix AEL, Montreal, Quebec) were used in series following a guard column containing Perisorb A (E. Merck, Darmstadt, GFR). Isomers of zearalenol in urine were sometimes separated on a single reverse phase Partisil-10 ODS column of similar dimensions.

(d) *Chromatographic columns.*—Glass (1.45 × 25 cm) with Teflon stopper (Kontes Glass Co., Vineland, NJ).

(e) *Vortex mixer.*—Canlab deluxe mixer (Canlab, Toronto, Ontario).

(f) *Gas chromatograph–mass spectrometer.*—Sigma 3 gas chromatograph (Perkin-Elmer Ltd, Montreal, Quebec) combined via glass jet separator to VG 7070 mass spectrometer (VG Micromass Ltd, Winsford, UK). Glass chromatographic column (183 × 0.2 cm id) packed with 2.5% SE-30 silicone gum rubber on 80–100 mesh Chromosorb W HP (Chromatographic Specialities Ltd., Brockville, Ontario).

(g) *Derivatizing vials.*—100 μL Reacti-Vials (Pierce Chemical Co., Rockford, IL).

Reagents

(a) *Extraction solvents.*—Pesticide grade methylene chloride, hexane, methanol, and ethyl acetate (Fisher Scientific, Toronto, Ontario).

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(**b**) *Column chromatographic materials.*—Sephadex LH-20, 0.025–0.100 mm (Pharmacia Fine Chemicals, Dorval, Quebec). Benzene–methanol (85 + 15), both pesticide grade (Fisher Scientific).

(**c**) *HPLC mobile solvents.*—(*1*) Normal phase system: isooctane–$CHCl_3$–methanol (35 + 25 + 3). (*2*) Reverse phase system: methanol–water (65 + 35) (HPLC grade, Fisher Scientific).

(**d**) *Zearalenone standard solution.*—1 μg/5 μL methanol (IMC Chemical Group Inc., Terre Haute, IN).

(**e**) *Zearalenol standard solution.*—1 μg/5 μL methanol for both α- and β-isomers.

(**f**) *6′α-Acetoxyzearalane internal standard solution.*—1 μg/5 μL methanol.

(**g**) *β-Glucuronidase.*—2 mg/200 μL 0.1M sodium acetate buffer (pH 5.0). Enzyme activity 920 000 units/g (bovine liver type B-1, Sigma Chemical Co., St. Louis, MO).

(**h**) *GC–MS derivatizing reagent.*—Tri-Sil TBT (Pierce Chemical Co.).

Experimental Animals

Four female weanling Wistar rats (Woodlyn Laboratories Ltd, Guelph, Ontario) were fed a casein-based semipurified diet (3) supplemented with 250 μg crystalline zearalenone/g diet for 2 weeks. All animals were then killed and livers were removed for analysis. Six other rats were fed the casein diet without supplementation for 2 weeks, and then were intubated with zearalenone (10 mg/100 g body weight) and urine was collected for 48 h as previously described (4).

Incubation of Urine

Pipet 0.5 mL urine and 5.0 mL sodium acetate buffer (pH 5.0) into duplicate 30 mL tubes. Add 200 μL β-glucuronidase solution to one tube and 200 μL sodium acetate buffer to duplicate tube. Incubate 24 h at 37°C.

Synthesis of 6′α-Acetoxyzearalane

Acetylate 1.0 g α-zearalanol (IMC Chemical Group Inc., Terre Haute, IN) for 18 h in the presence of 8.0 mL pyridine and 5.0 mL acetic anhydride. Hydrolyze excess acetic anhydride by adding 40 mL water. Extract resulting triacetate 3 times with 40 mL $CHCl_3$. Wash $CHCl_3$ extract with 1N HCl and then with water. Evaporate washed extract under vacuum to form thick residue. Reflux residue 1 h in 100 mL piperidine (10). After cooling add 50 mL ethyl acetate and extract mixture with 50 mL 6N HCl until pH of water layer is >7.0. Evaporate ethyl acetate layer to dryness under vacuum. Crystallize product from methylene chloride plus hexane.

Internal standard 6′α-acetoxyzearalane was isolated as white needles with melting point of 130–131°C. This compares with literature values of 133–134°C (11). Spectral data included UV maxima at 264 nm (ε 10 300) and 302 nm (ε 4700), and molecular ion M^+ 364. Proton magnetic resonance (PMR, 60 MHz) determinations indicated ($CDCl_3$) 1.36 ppm (3H,d,12′-CH_3, J = 6 cps), 2.03 (3H,s,6′-OAc), 3.32 (2H,m,1′-H), 5.13 (2H,m,6′-H,10′-H), 6.22 (1H,d,3 or 5-H, J = 2.5 cps), 6.28 (1H,d,3 or 5-H, J = 2.5 cps), 7.26 (1H,s,4-OH), 12.13 (1H,s,2-OH).

Synthesis of α- and β-Zearalenols (6′α; 6′β-Hydroxyzearalene)

Dissolve 2.0 g zearalenone in 10 mL ethanol and dropwise add 0.5 g sodium borohydride in 15 mL ethanol. Let stand 2 h at 23°C, add 50 mL water, and extract products with $CHCl_3$. Separate isomeric alcohols by HPLC (Partisil-10 ODS column; methanol–water, 65 + 35) and recrystallize from methanol–water.

α-Zearalenol was isolated as white needles with melting point of 164.5–165.5°C (168–169°C (11)); molecular ion M^+ 320 (302/320 0.61, base peak 188). PMR spectra included (d_6-acetone) 1.40 ppm (3H,d,12′-CH_3, J = 6 cps), 3.80 (1H,m,6′-H), 4.98 (1H,m,10′-H), 5.69 (1H,m,2′-H), 6.21 (1H,d, 3 or 5-H, J = 2.5 cps), 6.56 (1H,d, 3 or 5-H, J = 2.5 cps), 7.17 (1H,d,1′-H, J = 16 cps). α-Zearalenol fluoresced strongly under longwave UV light when analyzed by TLC (12).

β-Zearalenol was isolated as white needles with melting point of 161.5–162.0°C, (174–176°C (11)); molecular ion M^+ 320 (302/320 0.74, base peak 188). PMR spectra included (d_6-acetone) 1.36 ppm (3H,d,12′-CH_3, J = 6 cps), 3.71 (1H,m,6′-H), 5.12 (1H,m,10′-H), 5.91 (1H,m,2′-H), 6.29 (1H,d, 3 or 5-H, J = 2.5 cps), 6.54 (1H,d, 3 or 5-H, J = 2.5 cps), 6.90 (1H,d,1′-H, J = 16 cps). β-Zearalenol exhibited minimal fluorescence on TLC when viewed under UV light.

The α and β assignments were made by comparison of fluorescence, polarity, and mass spectral data as described by Hagler et al. (13).

Preparation of Samples for Extraction

Urine.—Add 50 μg 6′α-acetoxyzearalane internal standard to each of the tubes containing urine.

Liver.—Thoroughly homogenize 3.0 g liver in 15.0 mL distilled water and heat in boiling water bath until color changes from red to pale brown (ca 10 min). Resuspend denatured liver by re-

homogenizing followed by thorough mixing with vortex mixer. Transfer 6.0 mL homogenate to 30 mL tube and add 4.0 μg 6′α-acetoxyzearalane internal standard.

Extraction

Add 10 mL methylene chloride to tubes containing urine or liver and blend thoroughly with vortex mixer. Centrifuge 5 min at 3020 × g. Remove lower layer (methylene chloride) and filter through small cotton plug in Pasteur pipet. Collect filtrate in 25 mL tube. Extract aqueous upper layer twice with 5 mL portions of methylene chloride. Combine the 3 methylene chloride fractions and evaporate to dryness under nitrogen. Redissolve residue in 2.5 mL hexane, add 2.5 mL 90% methanol, and mix thoroughly with vortex mixer. Separate phases in clinical centrifuge (3 min, ¾ maximum speed). Discard upper (hexane) layer and re-extract 2 more times with 2.5 mL hexane. Discard hexane layer and evaporate lower layer to dryness under nitrogen. Dissolve residue in 3.0 mL ethyl acetate, add 1.0 mL water, and mix thoroughly with vortex mixer. Separate phases in clinical centrifuge as described previously. Discard lower (water) layer, add 1.0 mL water, and repeat extraction of ethyl acetate twice. Thoroughly rinse centrifuge tube with ethyl acetate, filter washings through cotton plug in Pasteur pipet, and combine with original layer of ethyl acetate. Evaporate to dryness under nitrogen.

Cleanup

Cleanup was a modification of the method of Holder et al. (14): Equilibrate 9 g Sephadex LH-20 resin by soaking in benzene–methanol (85 + 15) for 3 h. Add Sephadex slurry to glass chromatographic columns until column height is 180 mm for liver samples or 150 mm for urine samples. Dissolve residue from extraction procedure in 0.5 mL benzene–methanol (85 + 15) and apply to top of column. Rinse flask 3 times with 0.5 mL benzene–methanol and add rinse to top of column. Wash column with benzene–methanol (85 + 15) and collect eluate in 2.5 mL fractions. Discard first 7 fractions and collect fractions 8–13 for determination of liver zearalenone. Pool fractions 14–20 for determining isomers of zearalenol. Regenerate column by washing with 3 times the void volume of benzene–methanol. Evaporate samples under nitrogen and redissolve in 2 mL isooctane–$CHCl_3$–methanol (35 + 25 + 3). Discard first 5 fractions for urine samples, individually collect fractions 6–12 and pool fractions 13–20. Proceed as for liver samples.

Liquid Chromatography

Inject 2–200 μL sample, depending on concentrations of zearalenone and metabolites. Set absorbance detector to 280 nm and solvent flow rate to 1.5 mL/min with column pressure of ca 750 psi. Set chart speed at 1 cm/min. If necessary, improve resolution of zearalenol isomers in urine samples by using the reverse phase system. Evaporate samples under nitrogen and redissolve in 2.0 mL methanol–water (65 + 35) and apply to column with flow rate of 1.0 mL/min.

Gas Chromatography–Mass Spectrometry (GC-MS)

Collect zearalenone and zearalenol peaks directly from HPLC system and concentrate in 100 μL Reacti-Vials. Add 10 μL Tri-Sil TBT to sample and let stand 30 min at room temperature. Inject 1 μL into chromatograph with helium flow rate of 30 mL/min, injection block temperature 280°C, temperature program from 180 to 280°C at 5°/min, and interface temperature of 300°C. Operate mass spectrometer at 70 eV.

Results and Discussion

The method described permits the determination of both free and glucuronide-bound forms of zearalenone and zearalenol isomers in biological fluids and tissues. The limits of detection for the instrument are about 2.0 ng for zearalenone and 5.0 ng for zearalenols. The sensitivity of the technique for the described procedure and injection volumes is 270 ng zearalenone and 500 ng zearalenol/mL urine. Sensitivity for liver samples is 28 ng zearalenone and 107 ng zearalenol/g tissue. Higher sensitivities can be achieved by concentrating samples.

A normal phase HPLC tracing of an external standard mixture is given in Figure 1; chromatograms of zearalenone and isomers of zearalenol in rat urine are shown in Figures 2 and 3, respectively. This type of system uses a polar column and a relatively nonpolar solvent. Retention times of compounds, therefore, increase with polarity. The least polar compound, 6′α-acetoxyzearalane, eluted first followed by zearalenone, α-zearalenol, and β-zearalenol. In some urine samples, the resolution of β-zearalenol was confounded by the interference of an unidentified peak. Subsequent analysis of these samples by using a reverse phase system eliminated this interference. The elution pattern was

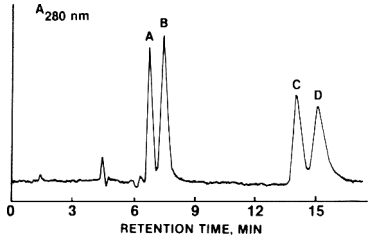

Figure 1. High pressure liquid chromatogram of a standard solution containing 100 ng 6′α-acetoxyzearalane (A), 50 ng zearalenone (B), 50 ng α-zearalenol (C), and 50 ng β-zearalenol (D). Conditions: normal phase Partisil-10 columns with isooctane-$CHCl_3$-methanol (35 + 25 + 3) mobile phase.

therefore reversed with β-zearalenol eluting before α-zearalenol (Figure 4). The 6′α-acetoxyzearalane internal standard could not be used with the reverse phase system because of overlap with the zearalenone peak. This made it necessary to first analyze samples with the normal phase system before using the reverse phase system.

Values for free and bound zearalenone and 6′α- and 6′β-zearalenols in urine are given in Table 1. In all cases the free form of excreted compounds exceeded bound forms by at least 100%. Variability between animals was increased because of difficulties in ensuring total collection of urine over the 48 h collection period. Free and bound forms of zearalenone and

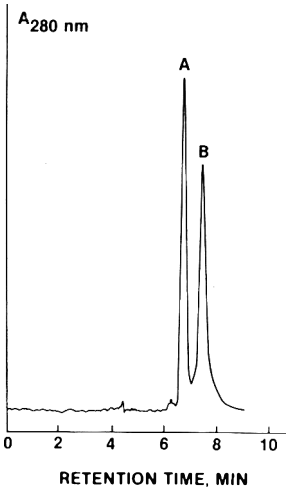

Figure 2. High pressure liquid chromatogram of urine from rats orally dosed with zearalenone: 6′-α-acetoxyzearalane internal standard (A) and zearalenone (B). Conditions: normal phase Partisil-10 columns with isooctane-$CHCl_3$-methanol (35 + 25 + 3) mobile phase.

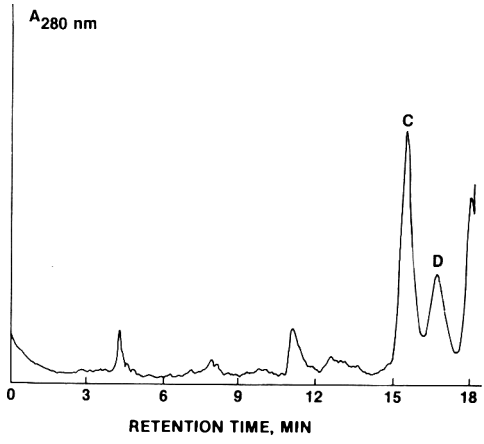

Figure 3. High pressure liquid chromatogram of urine from rats orally dosed with zearalenone: Tracing shows 6′α-zearalenol (C) and 6′β-zearalenol (D). Conditions: normal phase Partisil-10 columns with isooctane-$CHCl_3$-methanol (35 + 25 + 3) mobile phase.

metabolites have also been reported in rat urine by Ueno et al. (15), although the ^3H-labeling technique did not permit quantification. The finding that zearalenone is metabolized mainly to glucuronide conjugates and to a lesser degree to free or bound zearalenol agrees with previous reports (8).

Residue levels of zearalenone and metabolites in rat liver were determined only in free form. HPLC tracings of zearalenone and isomers of zearalenol are shown in Figures 5 and 6, respectively. Mean levels for 4 rats ± standard error for zearalenone, α-zearalenol, and β-zearalenol were 3.86 ± 0.60, 1.19 ± 0.12, and 0.23 ± 0.03 μg/g liver, respectively. Variability was less than that observed for urine samples because of more accurate sampling. These data confirm that in vivo conversion of zearalenone to zear-

Table 1. Zearalenone and metabolites in urine of rats orally dosed with zearalenone [a]

Compound	Free form	Glucuronide conjugates	Bound/free
Zearalenone	209.6	95.0	0.45
SEM	57.1	40.0	
α-Zearalenol	34.5	11.4	0.33
SEM	14.8	3.7	
β-Zearalenol	19.0	1.5	0.08
SEM	16.5	0.9	
Total	263.1	107.9	0.41
SEM	75.6	39.8	

[a] μg excreted in 48 h following dosing; mean ± standard error for 6 rats.

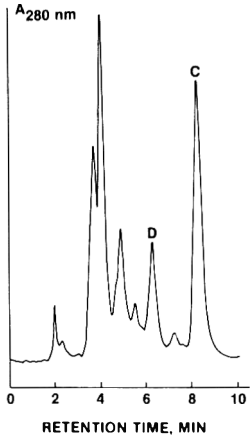

Figure 4. High pressure liquid chromatogram of urine from rats orally dosed with zearalenone: 6′-α-zearalenol (C) and 6′β-zearalenol (D). Conditions: reverse phase Partisil-10 ODS column with methanol-water (65 + 35) mobile phase.

alenol is likely of minor quantitative importance, although physiological significance may be greater.

Shreeve et al. (16) have reported transfer of zearalenone into urine and milk of cows but their methodology did not permit determination of zearalenol. Determinations of zearalenone in porcine and bovine milk have included HPLC methodology to detect both zearalenone and zearalenols (17, 18); however, internal standards were not included.

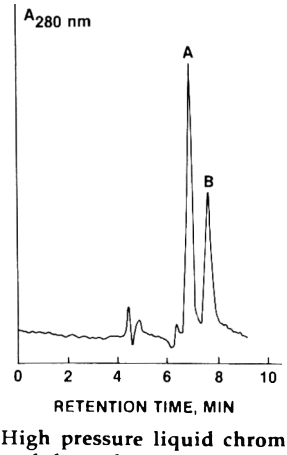

Figure 5. High pressure liquid chromatogram of liver from rats fed zearalenone: 6′α-acetoxyzearalane internal standard (A) and zearalenone (B). Conditions: normal phase Partisil-10 columns with isooctane-$CHCl_3$-methanol (35 + 25 + 3) mobile phase.

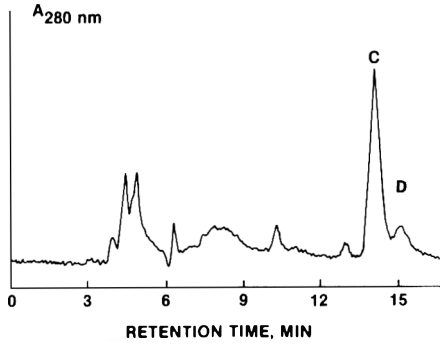

Figure 6. High pressure liquid chromatogram of liver from rats fed zearalenone: 6′α-zearalenol (C) and 6′β-zearalenol (D). Conditions: normal phase Partisil-10 columns with isooctane-$CHCl_3$-methanol (35 + 25 + 3) mobile phase.

The use of 6′α-acetoxyzearalane as an internal standard was very useful in improving reproducibility of analyses, because recovery values were inconsistent (50–80%). Thermal denaturation of liver was required before addition of the standard to prevent enzymic cleavage of the acetate group. An attempt to use an acid–base cleanup procedure was similarly unsuccessful because the internal standard was destroyed in the presence of base.

The described method permits rapid and sensitive determination of zearalenone and metabolites in biological fluids and tissues and should prove most successful in detection of residues in food products.

Acknowledgments

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References

(1) Funnell, H. S. (1979) *Can. J. Comp. Med.* **43,** 243–246
(2) Nelson, G. H., Christensen, C. M., & Mirocha, C. J. (1973) *J. Am. Vet. Med. Assoc.* **163,** 1276–1277
(3) Smith, T. K. (1980) *J. Anim. Sci.* **50,** 278–285
(4) Smith, T. K. (1980) *Can. J. Physiol. Pharmacol.* **58,** 1251–1255
(5) Patterson, D. S. P., & Roberts, B. A. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1265–1267
(6) Scott, P. M., Panalaks, T., Kanhere, S., & Miles, W. F. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 593–600
(7) Möller, T. E., & Josefsson, E. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 789–792

(8) Kiessling, K.-H., & Pettersson, H. (1978) *Acta Pharmacol. Toxicol.* **43,** 285-290
(9) Trenholm, H. L., Warner, R. M., & Farnworth, E. R. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 302-310
(10) Fishman, J. (1959) *J. Chem. Soc.* 1213-1216
(11) Shipchandler, M. T. (1975) *Heterocycles* **3,** 471-520
(12) Ueno, Y., & Tashiro, F. (1981) *J. Biochem.* 89, 563-571
(13) Hagler, W. M., Mirocha, C. J., Pathre, S. V., & Behrens, J. C. (1979) *Appl. Environ. Microbiol.* **37,** 849-853
(14) Holder, C. L., Nony, C. R., & Bowman, M. C. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 272-278
(15) Ueno, Y., Ayaki, S., Sato, N., & Ito, T. (1977) *Ann. Nutr. Aliment.* **31,** 935-948
(16) Shreeve, B. J., Patterson, D. S. P., & Roberts, B. A. (1979) *Food Cosmet. Toxicol.* **17,** 151-152
(17) Palyusik, M., Harrach, B., Mirocha, C. J., & Pathre, S. V. (1980) *Acta Vet. Acad. Sci. Hung.* 28, 217-222
(18) Hagler, W. M., Danko, G., Horvath, L., Palyusik, M., & Mirocha, C. J. (1980) *Acta Vet. Acad. Sci. Hung.* **28,** 209-216

METALS AND OTHER ELEMENTS

Gas Chromatographic-Atmospheric Pressure Active Nitrogen Method for Organomercury Speciation in Environmental Samples

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Methods are presented for the determination of methylmercury in fish, water, urine, and sediments, and diorganomercury compounds in water. Two significant differences from previous methods are the use of methylene chloride extracting solvent which permits sample extracts to be concentrated to volumes as low as 0.1 mL, and use of a gas chromatograph interfaced to an atmospheric pressure active nitrogen (APAN) afterflow detector. Such a detector is very sensitive and selective for Hg at picogram levels. Thus, limits of detection were significantly enhanced.

Organomercury compounds have considerably higher toxicity and longer biological half-lives in organisms than do inorganic forms of the element (1). Methylmercury and dimethylmercury are now known to be readily formed by bacterial biomethylation of inorganic mercury (2). Of these 2 compounds, methylmercury has been the predominant species found in biological systems, although the presence of dimethylmercury as well as other diorganomercury compounds and organomercury salts is possible (3). The widespread industrial use and natural distribution of mercury in the environment has made it imperative to develop analytical methodology for the specific determination of organomercury compounds which may originate from these sources.

In the past 25 years, numerous incidences of methylmercury poisoning from contaminated foods, particularly fish, have resulted in a number of methods for the specific determination of methylmercury in fish and other environmental samples. Early work by Westöö (4, 5) consisted of liberating the protein-bound methylmercury as methylmercury chloride by the addition of HCl. The CH_3HgCl was extracted into benzene and then back-extracted into an aqueous cysteine solution as a Hg–cysteine complex to eliminate interferences from benzene-soluble organic constituents in the subsequent gas chromatographic (GC) determination. The aqueous layer was re-acidified with HCl and the liberated CH_3HgCl was extracted back into benzene. The final analysis was performed by GC; an electron capture detector was used to measure the eluted CH_3HgCl. Although the Westöö procedure, with some modifications, has been recommended by the U.S. Environmental Protection Agency (6), it is a time-consuming procedure. Because the choice of solvents is limited by the use of an electron capture detector, the possibility of using other solvents, e.g., chlorinated hydrocarbons, is not feasible. Partitioning in each extraction is also not quantitative. Thus, correction factors are required in the calculations.

A method developed by Watts et al (7) eliminates the cysteine cleanup by using a simple acetone wash of the fish tissues to remove lipids and other organic molecules that may interfere in the GC analysis. The sample is then acidified with HCl and the liberated CH_3HgCl is extracted into benzene for analysis. The Watts procedure, although much simpler, is still susceptible to interferences in the chromatographic analysis from organic species not removed in the acetone wash but co-extracted into benzene with the CH_3HgCl.

In both the Westöö and Watts procedures, the ultimate limits of detection are constrained primarily by the volume of benzene used in the extractions. Attempts to concentrate the CH_3HgCl by evaporation of the benzene solvent results in losses of CH_3HgCl. It has been observed that the total volume of benzene must be kept at 8 mL or greater to avoid loss of CH_3HgCl (7).

A method described by Talmi (8) specifies a highly selective and sensitive microwave-induced plasma (MIP) as a GC detector for moni-

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toring Hg emissions at 253.7 nm. Samples are homogenized in water and acidified with HCl, and the CH_3HgCl is partitioned into 2 mL benzene for analysis. The selective detection of atomic mercury emissions eliminates interferences from co-extracted organic species if the GC is capable of resolving other organomercury compounds which may be present in the sample. Although the method is rapid and very sensitive, microwave plasmas have several operational limitations (8–10). The injection of microliter amounts of species into the plasma, i.e., solvent plug, can decouple the microwave resonant cavity, resulting in total quenching of the discharge. Thus, experimental manipulations are required to remove the solvent from the plasma before or after the species to be determined has eluted from the GC column. A limitation of MIP sources maintained at low pressure is the buildup of carbonaceous deposits on the walls of the discharge tube from effluents containing carbon; thus, periodic cleaning is required.

Methods for the analysis of surface waters for diorganomercury compounds have been very limited. A procedure developed by Dressman (11) involved extraction of several dialkylmercury species with a mixture of 20% ether in *n*-pentane, followed by concentration to 5 mL. The extract was then analyzed by gas chromatography with a flame ionization detector. The extractions were not quantitative and varied for each compound. The possibility of co-extracting organic interferences severely limits the applicability of the technique.

We have recently shown that an atmospheric pressure, active nitrogen (APAN) afterglow can function as a sensitive detector for several elements (12). Reactions of a variety of inorganic and organic species with active nitrogen sources have been extensively documented over several decades (13). In such reactions, the transfer of excitation energy from relatively long-lived metastable atomic and molecular nitrogen to species introduced in the discharge afterglow results in fragmentation and excitation of the introduced molecules, to produce characteristic atomic and/or molecular emissions.

Our recently reported observations (12) that the detection limit of Hg in the APAN afterglow was about 10 pg suggested an evaluation of this excitation source as a specific detector for the GC speciation of Hg. We report here the results of applying a GC-APAN system to the determination of methylmercury in fish, water, urine, and sediments, and diorganomercury compounds in water. The procedures described overcome several limitations or problems encountered in earlier methodologies for performing these determinations.

METHOD

Principle

Methylmercury is isolated from samples as methylmercury chloride and extracted into methylene chloride. Diorganomercury compounds are extracted from water with either methylene chloride or carbon disulfide. All extracts are concentrated to 1.0 mL, from which 2 μL aliquots are taken for analysis by gas chromatography. Interferences from other co-extracted organic molecules in the GC detection and quantitation of the organomercury species are eliminated by observing the Hg (I) emission at 253.7 nm excited in an APAN afterglow.

Reagents

(a) *Methylene chloride.*—Distilled in glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442). *Carbon disulfide.*—"Photrex" reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ 08865). No Hg was detectable when 50 mL of either solvent was evaporated to 1.0 mL and analyzed.

(b) *HCl solutions.*—Dilute HCl (ACS grade) to 6N in de-ionized water for sample acidifications.

(c) *Organomercury reference samples.*—Methylmercury chloride (Alfa Products, Danvers, MA 01923): Prepare stock solutions containing 1.0 mg/mL methylene chloride. Prepare serial dilutions containing concentrations of 100–0.01 μg/mL in 10 mL volumetric flasks.

Dimethylmercury, diethylmercury, di-*n*-propylmercury, diisopropylmercury, dibutylmercury, dihexylmercury, and diphenylmercury (Pfaltz and Bauer, Stamford, CT 06902): Prepare stock solutions containing 1.0 mg/mL of each diorganomercury species in ethyl ether as a mixture. Prepare serial dilutions in the same manner used for CH_3HgCl solutions.

Apparatus

(a) *Glassware.*—Standard filters, vacuum flasks, and separatory funnels. A specially designed flask, consisting of a 2.0 mL graduated tip on the bottom of a 50 mL round-bottom flask, was used for concentrating organic extracts (14).

(b) *Gas chromatograph.*—Hewlett-Packard Model 5700A was modified for interfacing to APAN afterglow by using heated transfer line. APAN instrumentation, excitation characteristics of afterglow, and details of interfacing to GC

system have been described previously (12, 15).

(c) *GC columns and conditions.*—(A) Methylmercury chloride: 4 ft × 1/8 in. od glass column containing 10% SP2401 on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA 16823); column temperature, 180°C; nitrogen flow rate, 110 mL/min; interface temperature, 230°C. (B) Diorganomercury compounds: 3 ft × 1/8 in. od stainless steel column containing 4% SE-30/6% SP2401 on 100–120 mesh Supelcoport (Supelco, Inc.); column temperature, 50°–210°C at 30°/min; nitrogen flow rate, 80 mL/min; interface temperature, 210°C. Before use, condition columns 24 h at 250°C at a nitrogen flow rate of 10 mL/min, followed by deactivation with 4 sequential 10 μL injections of silanizing reagent over a 1 h period.

(d) *Detector characteristics.*—APAN input power, 500 watts; nitrogen flow rate, 30 L/min; analytical wavelength, 253.7 nm; bandpass, 2 nm; PMT voltage, 850 V.

Isolation of Mercury Species from Samples

(a) *Methylmercury in fish.*—Prepare homogeneous samples of fish tissue by cutting ca 2.5 cm wide strip from head to tail. Finely chop tissue and then pulverize in stainless steel tissue grinder (Latapie Type, Arthur H. Thomas Co., Philadelphia, PA 19105). Transfer 1.0 g representative sample to 5 cm diameter, medium porosity filter and wash with four 15 mL portions of acetone, followed by two 15 mL portions of methylene chloride to remove water and lipids from sample, as described by Watts et al. (7). Remove each washing by vacuum filtration, and discard. Quantitatively transfer sample to 50 mL screw-cap bottle, add 20 mL portion of 6N HCl, and vigorously shake mixture 2 min. Add 20 mL methylene chloride to extract and again shake bottle an additional 2 min. Vacuum-filter entire contents to remove tissue and to break up any emulsions that may have formed. Separate organic layer from aqueous layer in 60 mL separatory funnel. Recombine tissue and aqueous layer in screw-cap bottle, add second 20 mL portion of methylene chloride, and repeat extraction. Combine organic fractions and concentrate to 1.0 mL on steam bath for subsequent analysis.

(b) *Methylmercury in water.*—Acidify 100 mL water sample with 20 mL 6N HCl and extract twice with 20 mL portions of methylene chloride in 250 mL separatory funnel. Separate organic layers from aqueous layers. Combine and concentrate organic fractions to 1.0 mL for analysis.

(c) *Methylmercury in urine.*—Follow procedure for water except use only 25 mL sample and add 10 drops of concentrated HCl to acidify sample. Vacuum-filter combined organic and aqueous layers to eliminate emulsions before separation.

(d) *Methylmercury in sediments.*—Contamination of sediments was expected to be a surface phenomenon; thus, no breakdown of the sediment particles was attempted. Dry sediment before analysis. Treat 1.0 g sample with 10 mL 6N HCl and extract with 20 mL methylene chloride in 50 mL screw-cap bottle Remove sediment by vacuum filtration. Separate organic layer and concentrate to 1.0 mL for analysis.

(e) *Diorganomercury compounds in water.*—Extract 1 L water sample three times with 20 mL portions of methylene chloride or carbon disulfide in 2 L separatory funnel. Combine organic fractions and concentrate to 1.0 mL for analysis.

Recovery Studies

Spike samples by adding 100 μL aliquots from reference CH_3HgCl solutions of appropriate concentration to give methylmercury concentrations of 1.0 and 10 ppb in water samples, 1.0 ppb in urine samples, and 1.0 and 10 ppm in sediment samples. Spike fish samples known to contain methylmercury with 2.0 or 20 ppm CH_3HgCl after removal of water from fish tissue by acetone washings as described by Watts et al. (7).

For diorganomercury compounds, spike 1 L water sample with 1.0 mL reference mixture containing 1 μg/mL of each compound to give concentration in water of 1.0 ppb (1 μg/L).

Results and Discussion

The APAN afterglow was a sensitive GC detector for Hg species monitored at the atomic mercury line at 253.7 nm. Detection limits were as follows: 2 pg dimethylmercury, 5 pg diethylmercury, 10 pg dipropylmercury, 20 pg dibutylmercury, 30 pg dihexylmercury, 50 pg diphenylmercury, and 50 pg methylmercury chloride. Values represent the absolute amounts of analyte required to give a peak height with signal-to-background scatter of three. Analytical calibration curves were linear to approximately 1 μg, well within the range of values expected from analysis of contaminated samples.

The 10% SP2401 column packing gave excellent and reproducible peak shapes for CH_3HgCl

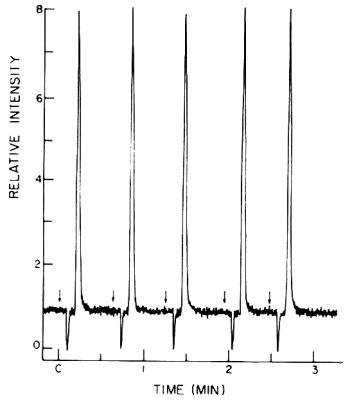

Figure 1. Chromatographic peaks obtained from 5 sequential 2 μL injections each containing 2 ng CH_3HgCl. Column temperature: 180°C. Arrows indicate point of sample injection.

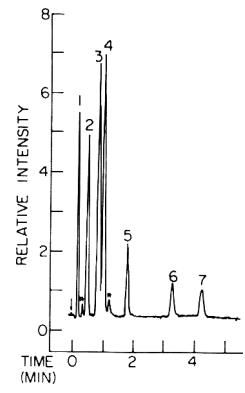

Figure 2. Chromatogram obtained from mixture of several diorganomercury species present at 2-5 ng levels. (1) $(CH_3)_2Hg$, (2) $(C_2H_5)_2Hg$, (3) $(i\text{-}C_3H_7)_2Hg$, (4) $(C_3H_7)_2Hg$, (5) $(C_4H_9)_2Hg$, (6) $(C_6H_{13})_2Hg$, (7) $(C_6H_5)_2Hg$, and (*) impurities. Temperature program: initial 50°C, rate 32°C/min, final 210°C with 2 min hold.

as shown in Figure 1 for 5 sequential 2 ng injections. The negative deflection before each peak was caused by the elution of the solvent, which temporarily caused quenching of the spectral background from the afterglow. This quenching effect had no effect on emissions from the sample effluents. An attempt was made to use column packings recommended by others for separating CH_3HgCl, including 15% DEGS (7) and 10% FFAP (8), but these packings gave considerably broader and tailing peaks.

A 5% SE-30/6% SP2401 column was excellent for separating a number of diorganomercury compounds, as shown by the chromatogram in Figure 2. The resolving power of the column is highlighted by the separation of the di-*n*-propyl and diisopropyl isomers of Hg.

The results of the analyses and recovery studies for methylmercury in fish, water, sediments, and urine are given in Table 1. The first 3 fish species were obtained from Canadian lakes suspected of having high levels of Hg contamination.

The precision of the determinations was acceptable, with deviations from the average value of 5% or less obtained from duplicate samples in all cases. An average recovery of 95% was obtained from samples spiked with known amounts of CH_3HgCl.

There appeared to be no loss of protein-bound methylmercury due to evaporation during the pulverization and washing of the fish tissues, or due to adsorption during the short contact time with the tissue grinder. There were also no mercury species detected in the solvents used for the preliminary washing of the fish tissue.

Removing emulsions by centrifugation did not improve recoveries. Apparently negligible amounts of CH_3HgCl were trapped in the emulsified material that was filtered from the urine and fish extractions.

Chromatograms of the samples in all cases exhibited only the CH_3HgCl peak. Organic

Table 1. Analysis and recovery study results for methylmercury in various sample types[a]

Sample	Found, ppm	Added	Recovered	Recovery, %
Walleye	10.2 (±0.3)	20.0 ppm	29.3 (±1.2) ppm	95
Lake trout	1.65 (±0.03)	2.0 ppm	3.55 (±0.13) ppm	95
Small mouth bass	4.04 (±0.07)	—	—	—
Carp	0.34 (±0.01)	—	—	—
Surface water	ND[b]	1.0 ppb	0.96 (±0.02) ppb	96
	ND	10 ppb	9.4 (±0.4) ppb	94
Sediments	ND	1.0 ppm	0.94 (±0.01) ppm	94
	ND	10 ppm	9.4 (±0.2) ppm	94
Urine	ND	1.0 ppb	0.96 (±0.02) ppb	96

[a] All duplicate samples.
[b] Not detected.

Table 2. Percent recovery of diorganomercury compounds from 1 L water samples, column adsorption and solvent extraction techniques

Compound[a]	Column recovery, %[b]	Solvent extraction recovery, %	
		CH_2Cl_2	CS_2
Dimethylmercury	15	23	25
Diethylmercury	25	98	98
Dipropylmercury	28	88	94
Dibutylmercury	35	85	99
Dihexylmercury	30	97	95
Diphenylmercury	80	99	98

[a] 1.0 ppb of each compound (1 μg/L).
[b] Methylene chloride used as eluting solvent.

Table 3. Detection limits for organomercury compounds in various sample types

Sample	Detection limits[a]	
	A, ppb	B, ppt
Diorganomercury in:		
Water, 1.0 L	0.01–0.05	0.4–4
Methylmercury in:		
Water, 100 mL	0.50	0.4
Urine, 25 mL	2	16
Sediments, 1.0 g	50	400
Fish, 1.0 g	50	400

[a] A = Extracts concentrated to 1.0 mL; 2 μL GC aliquots used. B = Sample quantity increased 5-fold, extracts concentrated to 0.1 mL, and 5 μL GC aliquots used.

materials present in the concentrated extracts appeared to have no detrimental effects on the column or detector performance. For example, peak heights of reference samples injected before and after 60 injections of several fish samples exhibited less than 5% deviation.

Benzene, ethyl ether, and carbon disulfide were also evaluated as solvents. Benzene was eliminated because of difficulties in concentrating extracts to small volumes (≤1 mL) without loss of CH_3HgCl. Ethyl ether was inadequate because of poor partitioning of CH_3HgCl due to co-extraction of HCl. Peak heights obtained from reference CH_3HgCl solutions prepared in carbon disulfide were only approximately one-fourth the peak heights obtained from identical reference solutions prepared in either benzene, ethyl ether, or methylene chloride. The reasons for this observed decrease in signal are unknown at this time.

Methylene chloride was the most suitable solvent of those evaluated. CH_3HgCl was efficiently extracted from acidified aqueous solutions with no apparent decomposition during chromatographic separations. Extracts could be concentrated to as little as 0.1 mL without loss of CH_3HgCl.

Recoveries for diorganomercury compounds at 1.0 ppb in water for 2 separation methods are given in Table 2. First, an attempt was made to elute water samples through an XAD-2 resin to adsorb Hg species on the XAD-2 surface. The Hg species were then eluted with 30 mL of methylene chloride and the solvent concentrated to 1.0 mL for chromatographic analysis. The dialkylmercury species, however, behaved in a similar manner observed for hydrocarbons: low recoveries due to poor adsorption on XAD-2 resin (G. Junk, Ames Laboratory, Iowa State University, private communication).

Multiple extractions with methylene chloride or carbon disulfide gave good recoveries, as indicated, for all diorganomercury compounds except dimethylmercury. The low recoveries suggest the latter compound is lost in the solvent concentration step because of its high volatility. Carbon disulfide gave the best extraction efficiency for the compounds studied. The reasons for the consistently lower recoveries for dipropyl and dibutyl Hg species when methylene chloride is used as the solvent are not known.

The detection limits obtained for several organomercury species, in reference to the actual water, urine, sediments, and fish samples studied, are summarized in Table 3. The detection limits in Column A are based on the injection of 2 μL aliquots from a 1.0 mL solvent concentrate for the given sample quantity. The detection limits can be significantly enhanced, as shown in Column B, if sample sizes are increased 5-fold, if extracts are concentrated to 0.1 mL, and if a 5 μL GC aliquot is used.

In this communication we have presented relatively simple and highly sensitive methods for determining organomercury compounds in a variety of environmental samples. The APAN detector exhibits excellent sensitivity, linear analytical calibration curves covering several orders of magnitude, good stability, and high specificity. Extensive sample cleanup is reduced because species other than mercury present in extracted samples will not interfere in the chromatographic analysis. Several limitations or problems encountered in earlier methodologies have been overcome. These include: (a) the afterglow detector allows the use of methylene choride, rather than the more toxic and less volatile benzene as the solvent; (b) the methylene chloride significantly reduced evaporative losses during preconcentration of the analyte, a factor

which has allowed for detection limits in the various samples to be improved over existing methods; and (c) the operational problems caused by solvent plug overloading and carbonaceous deposit formation in the element-specific MIP detector are circumvented. These factors make GC–APAN a viable alternative for the determination of organomercury compounds in food and environmental samples.

Acknowledgment

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REFERENCES

(1) Miettinen, J. K., Rahola, T., Hattula, T., Rissane, K., & Tillander, M. (1971) *Ann. Clin. Res.* **3,** 116–122

(2) Jensen, S., & Jernelov, A. (1969) *Nature London* **223,** 753–754

(3) Fishbein, L. (1973) *Chromatography of Environmental Hazards, Volume II, Metals, Gaseous, and Industrial Pollutants,* Elsevier Scientific Publishing Co., New York, NY, Chap. 7

(4) Westöö, G. (1966) *Acta Chem. Scand.* **20,** 2131–2137

(5) Westöö, G. (1967) *Acta Chem. Scand.* **21,** 1790–1800.

(6) Thompson, J. F. (Ed.) (1974) *Manual of Analytical Methods for the Analysis of Pesticide Residues in Human and Environmental Samples,* U.S. Environmental Protection Agency, Research Triangle Park, NC, Section 1-13, A

(7) Watts, J. O., Boyer, K. W., Cortez, A., & Elkins, E. R., Jr. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 1226–1233

(8) Talmi, Y. (1975) *Anal. Chim. Acta* **74,** 107–117

(9) Quimby, B. D., Uden, P. C., & Barnes, R. M. (1978) *Anal. Chem.* **50,** 2112–2118

(10) McLean, W. R., Stanton, D. L., & Penketh, G. E. (1973) *Analyst* **98,** 432–442

(11) Dressman, R. C. (1973) *J. Chromatogr. Sci.* **107,** 472–475

(12) D'Silva, A. P., Rice, G. W., & Fassel, V. A. (1980) *Appl. Spectrosc.* **34,** 578–584

(13) Wright, A. N., & Winkler, C. A. (1968) *Active Nitrogen,* Academic Press, New York, NY, and references contained therein

(14) Junk G. A., et al. (1974) *J. Chromatogr.* **99,** 745–762

(15) Rice, G. W., Richard, J. J., D'Silva, A. P., & Fassel, V. A. (1981) *Anal. Chem.* **53,** 1519–1522

Differential Pulse Polarographic Determination of Total Iodine in Milk

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A differential pulse polarographic method for total iodine in milk is presented. Samples are ashed, sodium hypochlorite is added to oxidize iodide to iodate, and the product is mixed with sodium sulfite solution and measured by differential pulse polarography at −90 V against SCE. The standard deviation for 53 duplicate samples was 0.011. Average recovery for 10 or more samples at different levels was 98.6% with a standard deviation of 2.9%.

The accurate quantitation of iodine in food products is necessary to establish the amount of iodine consumption by the consumer and to determine what levels are necessary for good nutrition. Especially important in these respects is the accurate determination of iodine in milk because it is possible for an 8 oz glass of this product to supply the minimum daily requirement for iodine. Milk iodine values can also be important in alerting the producer to possible iodine toxicity in the dairy herd and to help the producer select proper feed supplements for good iodine nutrition. The introduction of iodine-based teat dips and sanitizers has caused some concern about the possible contamination of milk with iodophors and this has resulted in the need to monitor iodine levels in milk. For these reasons, the following method for measuring total iodine in milk was developed. The method is reliable, selective, and extremely simple to incorporate as a routine procedure in the analytical laboratory.

METHOD

Apparatus

(a) *Differential pulse polarograph.*—EG&G Princeton Applied Research Model 174 polarographic analyzer.

(b) *Recorder.*—Houston Model 2000 with type 3 plug-ins.

(c) *Saturated calomel reference electrode (SCE).*

(d) *Tapered capillary.*—For use as dropping mercury electrode.

(e) *Zirconium crucibles.*—B-J Enterprises, Inc., Albany, OR.

Reagents

(a) *Ash aid A.*—Dissolve 50 g sodium nitrate and 50 g potassium nitrate in 200 mL water.

(b) *Ash aid B.*—20% sodium carbonate and 20% potassium dihydrogen phosphate, mixed 1 + 1.

(c) *Standardized iodide solution.*—1000 ppm iodide.

(d) *Sodium hypochlorite.*—5% (J. T. Baker Chemical Co.).

(e) *Organic iodine reference standard.*—*o*-Iodobenzoic acid (Ultrex, J. T. Baker Chemical Co.).

(f) *Organic iodine standards.*—8-Hydroxy-7-iodo-5-quinolinesulfonic acid, 2′,4′,5′,7′-tetraiodofluorescein disodium salt.

(g) *Inorganic iodine standards.*—Potassium iodate, potassium periodate.

(h) *Sodium sulfite.*—Saturated solution.

Procedure

Sample preparation.—Weigh 2.00 g well mixed milk into adequately marked ashing vessel, add 0.5 mL ash aid A, mix well by gentle swirling, and dry on steam bath, usually about 3 h or overnight.

Calibration curve.—In the following procedure, ashing vessel 1 is used to establish reagent blank and ashing vessels 2–9 are used as standards to determine response factor of standard curve.

To each of 9 ashing vessels, add, respectively, 0, 0, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, and 4.00 μg iodide. To vessels 2–9, add 2.00 mL low iodine-content milk and mix well by gentle swirling. Treat reagent blank and standards exactly as samples, as given in *Sample preparation*, beginning ". . . add 0.5 mL ash aid A, . . ."

Ashing procedure.—Use muffle furnace large enough to allow about 1½ in. head space above ashing vessels. Place dry samples and standards in 100°C muffle furnace 1 h; then set temperature to 200°C with low control setting, and check periodically to ascertain if smoking has ceased. Set temperature to 250°C with low control set-

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ting; after smoking has ceased, set temperature to 350°C with medium control setting and leave samples at this temperature 1 h. Set temperature to 525°C with high control setting. Leave samples at final temperature until ash is white and no carbon particles are visible. This will usually happen immediately after the temperature has reached 525°C. Let samples cool in muffle to about 250°C, remove, and let cool.

Oxidation of iodide to iodate.—To each cooled sample and standard, add 6 mL water and 4 drops of 5% sodium hypochlorite solution. Heat moderately on steam bath until ash is dispersed throughout solution, heat additional 3 min, remove from steam bath, and let cool to room temperature.

Polarographic analysis.—Quantitatively transfer samples/standards to 10 mL graduated cylinder equipped with glass stopper, using at least 3 washings of ashing vessel with distilled water and keeping total volume between 8.5–9.5 mL. Dilute to 10.0 mL mark using saturated sodium sulfite solution, mix by inverting, and transfer to electrochemical cell. Perform polarographic analysis for samples and standards by using parameters given below:

Initial potential	−0.90 V against SCE
Mode	differential pulse
Modulation amplitude	50 mV
Drop time	2 s
Scan rate	2 mV/s
Scan direction	negative
Sensitivity	2 μamps full scale
Low pass filter	off

Calculations.—Measure peak height of samples and standards by extending level baseline beneath peak maximum, and measure distance, in μamps, between this baseline and peak maximum. Determine reagent blank response from ashing vessel 1, described in calibration curve section, and perform blank subtraction for each sample and standard.

Linear regression is recommended procedure for characterizing relationship between response, μamps peak height, and the standards, μg iodide added. Slope of linear regression equation is then used for response. Alternatively, plot standard curve of μamps peak height against added iodide, find slope, and use this as response. Total iodine in sample is then calculated as

$$\text{Iodine, ppm} = (S - B)/(R \times W)$$

where S and B = μamps peak height of sample and reagent blank, respectively; W = g sample weight; and R = response factor calculated from standard curve, μamps peak height per added μg iodide.

Discussion and Results

The method as written can accommodate milk sample sizes up to 3 g. Larger sizes may be used; however, the amount of ash aid A should be decreased to 0.2 mL and the ashing rate will have to be slower. If low-iodine milk samples are encountered, 0.05–0.2 ppm, a 5 g sample may be necessary. This will increase the response sufficiently for accurate measurement; however, the recoveries are only 85–92% and the precision is not as good as that when 2 g samples are used.

The iodine standard is in the form of iodide because this is the prevalent form present in normal milk and also is the major form present when milk is exposed to elemental iodine or iodophors (C. S. Gelda, E. L. Thomas, J. J. Jezeski, W. G. Mizuno, & E. D. Berglund (1962) *J. Dairy Sci.* **45**, 663).

There were no differences, at the 95% confidence level of significance, in the regression response factors calculated from each of the 20 standard curves used in the development work. This can be expected because the recoveries are nearly quantitative and the dropping mercury electrode has a self-calibrating capability due to its dependence on physical properties which may be duplicated from day to day. This would suggest that the standard curve need not be run for every batch of samples, although some controls would be necessary to validate the response.

The standard curve response factor is determined by using the peak height responses obtained from ashing vessels 2–9 and using linear regression analysis to calculate the ratio of μamps peak height per added μg iodide. By incorporating a low iodine-content milk sample in the standards, it is believed that actual sample conditions are simulated during the analysis. The disadvantage of doing this is that some very low iodine-content milk samples may not be bracketed by the standard curve. Alternatively, the standard curve may be prepared with only water standards and this will ensure that all sample responses are bracketed. If only water standards are used, then ashing vessels 1 and 2 are both reagent blanks and their peak height responses should be averaged to obtain the blank value. There seems to be no difference in the regression response factors found by incorporating a milk matrix in the standards or by using water: response slope of curve and 95% confidence in-

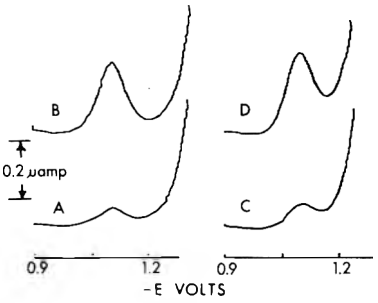

Figure 1. Differential pulse polarographic determination of iodate.

A, 0.1 ppm iodine, 5 g milk; B, 0.4 ppm iodine, 5 g milk; C, 0.3 ppm iodine, 2 g milk; D, 0.9 ppm iodine, 2 g milk.

terval for milk matrix = 0.141 μamp/μg I and 0.138–0.143; for water matrix = 0.143 and 0.136–0.150, respectively. It may be concluded that the difference is not significant.

The temperatures given in the ashing procedure, and the rate of increase to these temperatures, are critical in preventing the ignition of the samples. The ashing method is written for 2 g sample sizes. If sample sizes larger than 3 g are used, the amount of ash aid A should be decreased to 0.2 mL and only the low control setting used until the temperature reaches 400°C. The high control setting can then be used to increase the temperature to 525°C. Pyrex beakers, nickel crucibles, and zirconium crucibles have successfully been used for the ashing vessels. The zirconium crucibles are preferable.

The complete oxidation of iodide and sample dissolution is assisted by the heating of the sample after adding water and hypochlorite. Inadequate heating will result in poor recoveries. The sample is not completely dissolved by the heating and some precipitate will remain during the polarographic analysis. This does not interfere with the quantitation of iodate.

Illustrations of typical iodate peaks are shown in Figure 1. A greater sensitivity may be obtained by using the 100 mV pulse and this will shift the potential corresponding to the peak of the curve slightly more anodic than that shown in the figure.

No interferences have been detected. Iron is reduced at −0.75 V against SCE, and if present in high amounts it will cause a distorted baseline in the vicinity of the iodate peak. Nitrate is reduced at −1.55 V and if present in a large excess it will distort the iodate peak. This will not interfere with the quantitation of iodate. This nitrate effect will be noticed mainly when running samples low in organic material because the nitrate level is not greatly diminished during the ashing procedure. Curves C and D in Figure 1 show this distortion.

The polarographic method was used for the final detection of iodine for 2 reasons: (*1*) it greatly simplifies the method, (2) it provides a great deal of sensitivity. Other methods have been successfully used; however, they all required the incorporation of more reagents and/or the controlling of several more variables to obtain reliable results. This complicated the method and increased the analysis time.

The method was designed to completely analyze about 36 samples in an 8 h working day. This necessitated sacrificing some experimental parameters to shorten the procedure. If the analysis time is not critical, there are some advantages that may be gained by a simple modification of the method. By increasing the sample size to 5 g and decreasing the amount of ash aid A to 0.2 mL, the iodate curve is considerably improved. This is because of the reduced influence of the nitrate on the iodate peak shape due to less nitrate being added and because more nitrate is used in destroying the larger amount of organic material. The improvement can be seen in Figure 1 where curves A and B may be compared with curves C and D. The advantage of improving the curve is that this allows the use of the 100 mV pulse amplitude and this will significantly increase the sensitivity without sacrificing resolution. Also, if the drop time is then decreased to 0.5 s and the scan rate is increased to 5 mV per s, the sample through-put on the polarograph will be doubled without sacrificing sensitivity. A convenient result is that the calibration curve obtained by using the above parameters is equal to that obtained when using the parameters given in the procedure.

The standard deviation was 0.011 ppm iodine and was estimated from 53 duplicate samples. The detection limit, for a 2 g sample size, was 0.05 ppm iodine and was sufficient for the quantitation of iodine in all milks from the Michigan area. The average recovery of iodine was 98.6% with a standard deviation of 2.9 percent: Average recoveries for 10 or more samples at different levels were 98.8 ± 2.1, 98.1 ± 3.4, 98.2 ± 3.4, 98.8 ± 2.1, 96.7 ± 4.0, 100.0 ± 1.4, 97.3 ± 2.9, 100.0 ± 1.9, and 99.2 ± 2.3% for 0.50, 1.0, 1.50, 2.0, 2.50, 3.0, 3.50, 4.0, and 5.0 μg I.

Table 1 shows the recoveries of inorganic and organic iodine. The organic compound used as a reference standard was Ultrex grade *o*-iodobenzoic acid and was certified to be 99.9% pure.

Table 1. Inorganic and organic iodine recoveries

Iodine form	Range, μg I	No. of recoveries	Recovery, % ± SD
Potassium iodide	0.1–6	>100	98.8 ± 1.8
Potassium iodate[a]	0.5–4.5	20	98.8 ± 1.5
Potassium periodate	0.5–4.5	9	104.7 ± 1.9
8-Hydroxy-7-iodo-5-quinolinesulfonic acid	0.5–4.5	9	101.0 ± 1.8
2,4,5,7-Tetraiodofluorescein disodium salt	0.5–3	14	98.0 ± 2.0
o-Iodobenzoic acid[b]	0.5–3	17	101.0 ± 2.1

[a] Inorganic iodine reference standard.
[b] Organic iodine reference standard.

The inorganic reference standard was potassium iodate recrystallized 3 times from water and dried at 180°C. The nearly quantitative recoveries of both forms of iodine would suggest that the results should be expressed in total iodine units.

The range of iodine in raw milk from the Michigan area was 0.05–3.5 ppm with a mean of 0.35 ppm. The concentration of iodine in raw milk was quite variable and was assumed to reflect the amount of iodine fed to the cow. The use of iodophors, an iodine-based teat dip and sanitizer, may cause some elevation of iodine levels in milk; however, data from the regulatory laboratory has suggested that the extreme variation of iodine levels is primarily a result of iodine-supplemented feeds.

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PESTICIDE RESIDUES

Colorimetric Determination of 3-Amino-1,2,4-Triazole in Grain or Meal

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This colorimetric method for the determination of 3-amino-1,2,4-triazole in grain or meal is a modification of the Storherr and Burke method. The herbicide is extracted from grain with methanol, and purified by adsorption-desorption on resin. The extract is cleaned by digestion with sulfuric acid and clarified with charcoal. The colored complex formed by coupling with *N*-(1-naphthyl)ethylenediammonium dichloride is measured spectrophotometrically at 455 nm. The limit of detection is 0.05 ppm.

3-Amino-1,2,4-triazole (aminotriazole or amitrole) is a non-selective herbicide used before planting wheat, oats, maize, and kale. It is absorbed by roots and leaves and translocated (1).

The present colorimetric method for determining residues of amitrole in grain or meal is an adaptation of the method of Storherr and Burke (2, 3), and has been developed because the Storherr and Burke method is lengthy and gives poor reproducibility. The extraction step has been modified, the resin adsorption-desorption is well controlled, and cleanup is simplified. Color development has been particularly studied, and the limit of detection of the method has been lowered.

METHOD

Reagents

(a) *Activated charcoal.*—Boil and stir for 2 h on sand bath 120 g charcoal (Merck 2186), 100 mL water, and 300 mL concentrated H_2SO_4. Filter through large fritted glass Buchner funnel (pore size 90–150 μm), and wash residue on funnel with hot water until washings are neutral to universal indicator paper. Dry charcoal 48 h in 130°C oven, cool, and shake to powder. Store in closed container.

(b) *Filter aid.*—Celite 545 (Johns-Manville).

(c) *Ion exchange resin I.*—Pour 250 g resin (Merck 4765) onto large fritted glass Buchner funnel (pore size 90–150 μm), rinse with 2 L hot concentrated NH_4OH, filter under suction, and wash with hot water until washings are neutral to universal indicator paper. Then stir resin with 2 L hot 5N HCl, filter under suction, and wash with water until washings are neutral. Dry resin 2 h under suction and store in closed container at room temperature. Used resin can be reactivated by same procedure.

(d) *Standard.*—3-Amino-1,2,4-triazole (Pestanal Riedel- de Haën No. 35701, >99% pure).

(e) *Standard solutions.—Stock solution.*—100.0 mg 3-amino-1,2,4-triazole in 500 mL water. *Working solutions.*—Prepare appropriate dilutions in water as needed.

(f) *Sulfuric acid GR.*—Concentrated H_2SO_4–water (75 + 25).

(g) *Sodium nitrite GR.*—0.5% aqueous solution. Prepare fresh daily.

(h) *Ammonium sulfamate GR.*—Merck 1220. Prepare 5% aqueous solution.

(i) *N-(1-Naphthyl)ethylenediammonium dichloride GR.*—Merck 6237. Prepare 1% solution in 2 N HCl. Prepare fresh daily.

Extraction of Grain

Pulverize grain in chopper. In Omni-mixer, blend 100 g homogeneous meal with 20 g Celite filter aid and 250 mL methanol for 3 min at high speed. Transfer mixture to 500 mL centrifuge bottle and centrifuge 5 min at 3000 × g. Filter supernate on 10 cm diameter Buchner funnel through 1 cm layer of filter aid into 1 L Erlenmeyer flask. Re-extract residue in bottom of centrifuge tube with 150 mL methanol, centrifuge, filter, and wash filter aid with 50 mL methanol. Combine extracts and washings in same flask. Add 15 mL 30% H_2O_2. Heat 30 min on (200°C) sand bath; then cool at room temperature.

Purification on Resin

Add 30 g ion exchange resin (c) to flask containing methanol extract, stopper, and shake mechanically 45 min. Transfer resin and liquid onto 300 × 20 mm chromatographic column

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containing glass wool plug. Discard liquid. Wash resin with three 30 mL portions of acetone and with four 50 mL portions of water. Discard all washings.

Replace resin into previous flask, add 50 mL water and 20 mL concentrated NH_4OH, and boil 30 min on 200°C sand bath.

Transfer hot mixture onto same chromatographic column containing glass wool plug, and collect eluate and subsequent washings in 400 mL beaker. Wash flask and column with four 25 mL portions of 2N NH_4OH. Concentrate combined eluates and washings on sand bath to ca 20 mL, and cool.

Cleanup

Add 5 mL H_2SO_4–water solution (f) to cooled concentrated extract. Add 2 glass beads, cover with watch glass, and boil gently 10 min on 200°C sand bath. Cool this solution, add 50 mL water and 0.8 g activated charcoal, cover with same watch glass, and boil 15 min on sand bath.

Filter hot mixture on 4.5 cm diameter Buchner funnel through S&S No. 602 paper, or equivalent, and collect filtrate in 400 mL beaker. Wash reaction beaker and funnel with 200 mL hot 0.75% H_2SO_4, followed by 50 mL hot water.

Concentrate combined filtrates and washings to 15 mL. If solution, after that concentration, is yellowish in color, an additional charcoal cleanup is necessary. To do this, add 50 mL water and maximum 0.2 g charcoal to concentrated solution; heat to boiling 15 min, filter, and concentrate as described above.

Cool concentrate and dilute to 25 mL with water. Shake mixture thoroughly and filter through folded paper. Take 5 mL aliquots for color reaction.

Color Development

Pipet 5 mL sample aliquots into each of two 25 mL Erlenmeyer flasks. Add 1 mL water and 3 mL H_2SO_4–water solution, and swirl. Add 0.5 mL 0.5% $NaNO_2$ solution, swirl, and let stand ≥30 min. Add 0.5 mL 5% ammonium sulfamate solution, swirl, and immediately eliminate nitrogen oxides from solution by 0.5 min ultrasonic bath treatment, followed by manual stirring under light air current dispensed through Pasteur capillary pipet. Immediately, add to one sample flask 0.5 mL 1% *N*-(1-naphthyl)ethylenediammonium dichloride solution, and swirl. Elimination of nitrogen oxides and addition of color reagent must be done in less than 2 min. To other flask, add 0.5 mL water, and swirl. Let both flasks stand ≥5 min.

Measure absorbance of both solutions against water at 455 nm. Subtract blank from sample reading. With standard curve, determine amount of amitrole in the 5 mL aliquot.

Table 1. Effect of resin adsorption and desorption on recovery of 9.42 μg amitrole

Resin-conditioning treatment	Rec., μg	Rec., %
Cold 5% NaOH, cold 5 N HCl	7.33	77.8
Hot 5% NaOH, hot 5 N HCl	8.33	88.4
Cold concd NH_4OH, cold 5 N HCl	8.63	91.6
Hot concd NH_4OH, hot 5 N HCl	8.91	94.6

Calibration Curve

Into a series of 25 mL Erlenmeyer flasks, pipet 5 mL portions of appropriate working solutions containing 0, 2, 4, 8, 16, 24, and 32 μg amitrole. To each flask add 1 mL water and 3 mL H_2SO_4–water solution, and swirl. Add 0.5 mL 0.5% $NaNO_2$ solution. Swirl, and let stand 30 min. Then proceed as for sample determination. Draw standard curve and/or determine regression line and correlation factor, *r*.

Results and Discussion

Extraction

The filtration step in the extraction procedure of Storherr and Burke (2) has been replaced by centrifugation to reduce amitrole losses because filtration after blending results in considerable foam in the Buchner funnel, washing is not easy, and the extract is not very clear. These difficulties are not encountered in centrifugation, which allows an easier additional extraction. Moreover, this centrifugation is faster than the filtration step.

Purification on Resin

The purification described by Storherr and Burke (2) has been retained, except for the conditioning step of the resin and the quantity used. Table 1 shows the influence of conditioning of resin on recovery. Effect of the amount of resin used was tested on 9.42 μg amitrole. For 20, 25, 30, 35, and 40 g resin, recoveries were 8.38 (90.0), 8.90 (94.5), 8.92 (94.7), 8.91 (94.6), and 8.98 μg (94.8%).

Table 2. Effect of dilution on absorbance of colored solution at high amitrole concentrations

Amitrole, μg/5 mL	Direct absorbance	Water diln	Absorbance after diln
20.05	0.585	—	0.585
60.15	1.734	1/3	0.584
100.25	>2.500	1/5	0.510
200.50	>2.500	1/10	0.533
601.50	>2.500	1/30	0.510

Cleanup

The cleanup step is critical. When a poor quality of charcoal is used, the recovery and reproducibility of the method decrease considerably. It is important to use a freshly activated charcoal to ensure a recovery of 90% or better. Therefore, each new charcoal batch must be checked before use. For 9.42 μg amitrole and 0.8, 1.0, and 1.2 g charcoal, recoveries were 8.93 (95.3), 8.80 (93.4), and 8.22 μg (87.3%).

With the present method, the acetonitrile cleanup recommended by Storherr and Burke (2) is not necessary; in fact, the acetonitrile cleanup could reduce the sensitivity of the method.

Color Development

Color development is the most sensitive step of the method. The times between the addition of the various reagents in the color development sequence have to be carefully controlled.

After the addition of $NaNO_2$ solution, there must be a delay of at least 30 min before the sulfamate solution is added. In a study on the effect of time between addition of these 2 solutions on recovery of 9.25 μg amitrole, for 1, 10, 20, 30, 40, and 70 min periods between additions, absorbances were 0.240, 0.265, 0.268, 0.270, 0.270, and 0.270, respectively.

As soon as the sulfamate solution has been added, the delay for eliminating nitrogen oxides and for adding the color reagent must be as short as possible (<2 min). Studies on the recovery of 9.25 μg amitrole showed absorbances of 0.270, 0.266, 0.260, 0.254, 0.247, 0.239, and 0.200 for times of 0.5, 1, 2, 3, 4, 5, and 10 min, respectively, between ammonium sulfamate addition and elimination of nitrogen oxides. Similar studies on the effect of time between elimination of nitrogen oxides and addition of color reagent showed absorbances of 0.270, 0.270, 0.267, 0.255, 0.229, 0.188, and 0.148 for 0.5, 1, 3, 5, 10, 20, and 30 min periods, respectively.

Color development is complete after 5 min and the color developed is stable for a maximum of 30 min, after which fading is observed.

Calibration Curve

When the conditions for color development are correctly followed, the curve is linear between 2 and 32 μg amitrole/5 mL (0.10–1.6 ppm in grain under the conditions of the method), and its correlation factor is better than 0.9995.

For more concentrated solutions (up to 500 μg/5 mL, or 25 ppm), an estimation can be made by diluting the final colored solution with water. But, as shown in Table 2, an accurate reading is obtained only by repeating the entire determination, reducing the initial sample weight, or diluting the solution before color development in such a way that the absorbance is less than 0.900.

Table 3. Recovery of amitrole added to grain

Added, 1.004 mg/kg			Added, 0.183 mg/kg			Added, 0.05 mg/kg		
Rec., ppm (incl. blank)	Crop blank, ppm	Rec., % (corrected)	Rec., ppm (incl. blank)	Crop blank, ppm	Rec., % (corrected)	Rec., ppm (incl. blank)	Crop blank, ppm	Rec., % (corrected)
0.891	0.002	86.2	0.169	0.003	90.7	0.060	0.010	100
0.891	0.003	86.0	0.171	0.006	91.3	0.061	0.006	111
0.887	0.002	86.6	0.183	0.011	93.4	0.065	0.023	104
0.895	0.001	88.1	0.167	0.005	88.5	0.067	0.013	108
0.887	0.001	87.2	0.157	0.003	84.1	0.065	0.015	100
0.898	0.001	88.7	0.154	0.001	83.1	0.060	0.013	94
0.879	0.000	87.2				0.073	0.021	104
0.890	0.000	88.1				0.061	0.008	106
0.894	0.001	88.4				0.052	0.008	88
0.891	0.001	87.5				0.058	0.010	96
Mean rec.		87.5			88.6			101
Variance		0.814			14.201			43.690
Std dev.		1.027			4.257			6.610

Limits of the Method

Determinations on grain samples spiked at 1.004, 0.183, and 0.05 mg/kg showed recoveries of 87.5, 88.6, and 101%, respectively (Table 3).

The concentration limit for the colored reaction is 1 μg amitrole in 5 mL. Thus, based on the method and its crop blank, the limit of detection is 0.05 ppm. It is not suitable to try to increase the limit of detection by increasing sample weight, because when samples are larger than 100 g, the crop blank value increases and reproducibility and recovery decrease.

The increase of crop blank value from spiked grain at 1.004–0.05 mg/kg is due to the calibration curve which has not exactly the same slope at very low levels (2 μg amitrole/5 mL). However, the present method allows the determination, with good precision, of 0.05 ppm amitrole in grain.

The modifications of the Storherr and Burke method (extraction and cleanup) and the understanding of all the steps, particularly cleanup and color development, and their influence on the final result, have resulted in a method with improved sensitivity and reproducibility.

Acknowledgments

The authors thank Josiane Potvin and Daniel Berger for laboratory assistance.

References

(1) Martin, H., & Worthing, C. R. (1977) *Pesticide Manual*, British Crop Protection Council, p. 14
(2) Storherr, R. W., Burke, J. (1961) *J. Assoc. Off. Agric. Chem.* **44**, 196–199
(3) *Pesticide Analytical Manual* (1977) Vol. II, Food and Drug Administration, Washington, DC

Gas-Liquid Chromatographic Determination of Residues of Oxadiazon and Its Metabolites in Green and Dry Hops and Hop Foliage

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Ronstar EC (emulsifiable concentrate) was applied at rates of 4.48 or 8.96 kg ai/ha as a broadcast spray during the winter, in late spring, and in mid-summer to plots in commercial hop fields. Residues of oxadiazon, the active ingredient, and its 4 metabolites (methoxy, alcohol, acid, and phenol) were determined at harvest (1 month after last treatment) in green and dry hops and in hop foliage, by using gas-liquid chromatography after column chromatographic cleanup. Drying the cone increased residues of oxadiazon, but resulted in loss of the methoxy and alcohol metabolites. Oxadiazon residues in hop foliage ranged from 0.058 to 0.163 ppm. Measurable amounts of metabolites were also found in the foliage.

Oxadiazon (2-*tert*-butyl-4-(2,4-dichloro-5-isopropoxyphenyl) - Δ^2-1,3,4-oxadiazolin-5-one, Ronstar®) is an effective herbicide for control of obnoxious grasses and broadleaf weeds in a wide variety of crops. However, its use with a particular crop depends primarily on the amount of residue left in the harvested crop. Ambrosi et al. (1, 2) studied its persistence and metabolism in various types of soil, and in an aquatic model ecosystem, where the residue could be easily transferred through the biological chain. They found that oxadiazon degraded slowly in all soils tested. Hirata et al. (3, 4) did an extensive study on residues in rice. They found that oxadiazon accumulated in the lower leaves and stems of the rice plant, in shoots of seedlings, and in straw of plants at harvesting. Ishizuka et al. (4) found that oxadiazon was stable in rice plants, with 75–80% of the residue identified as oxadiazon. Guardigli (5) determined residues of oxadiazon and its metabolites in soybeans, peanuts, and their by-products. The major isolated residue was unchanged oxadiazon, with lesser amounts of the 4 unidentified metabolites. He reported the use of a Florisil column for eliminating interfering plant material. Bingham et al. (6) determined the uptake and metabolism in various parts of the peanut plant when it was grown in soil treated with oxadiazon, and found about 0.74% of the applied oxadiazon equivalent in the plant at maturity. They specified the use of thin layer chromatography for cleanup of the residue sample.

When it became apparent that oxadiazon would control obnoxious weeds in hop fields, the need arose for data on residues of oxadiazon and its methoxy, alcohol, acid, and phenol (metabolites) (Figure 1) in green and dried hops and in hop foliage. This paper reports on a gas-liquid chromatographic (GLC) method for determining these residues, following column chromatographic cleanup.

METHOD

Reagents and Apparatus

(a) *Solvents.*—All solvents were redistilled in an all-glass system.

(b) *Herbicide standard solutions.*—Prepare separate solutions of 1.0 μg oxadiazon, oxadiazon methoxy, oxadiazon alcohol, oxadiazon acid, and oxadiazon phenol (Rhône-Poulenc, Inc., Monmouth Junction, NJ 09952)/mL dichloromethane. Store solutions in refrigerator.

(c) *Silica gel.*—Baker Analyzed Reagent grade (No. 3405, J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

(d) *Aluminum oxide.*—Acid (J.T. Baker).

(e) *Boron trifluoride solution.*—14% BF_3 in methanol (Pierce Chemical Co., Rockford, IL 61105).

(f) *Gas chromatograph.*—Hewlett-Packard 5840A equipped with nitrogen-phosphorus selective detector. Operating conditions: temperatures (°C)—injector 250, column 240, detector 300. 1.22 m (4 ft) × 4.763 mm (3/16 in.) glass column packed with 80–100 mesh Chromosorb GHP coated with 5% OV-101; helium gas flow rate 60 mL/min.

Field Treatment

Ronstar EC (emulsifiable concentrate) (2 lb/gal.) was applied at 2 rates, 4.48 or 8.96 kg oxadiazon ai/ha (4 or 8 lb oxadiazon ai/acre, respectively), in 2–4 replicated plots in commercial hop yards at 4 different locations. Oxadiazon was applied as a broadcast spray to soil during

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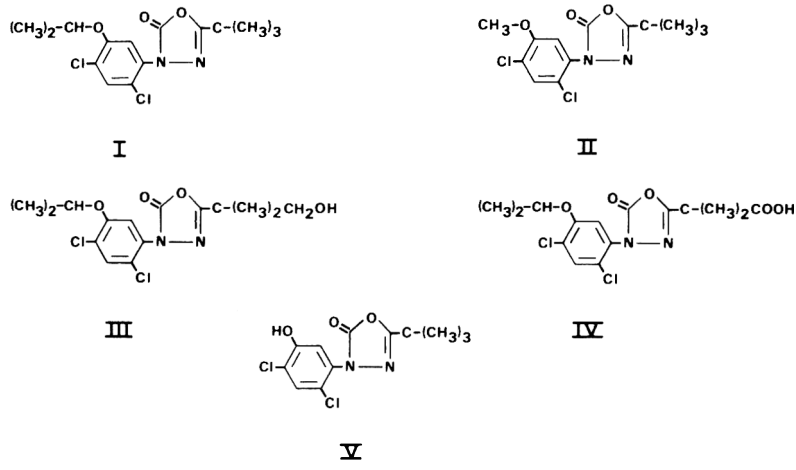

Figure 1. Structures of oxadiazon (I) and its metabolites, oxadiazon methoxy (II), oxadiazon alcohol (III), oxadiazon acid (IV), and oxadiazon phenol (V).

winter (or late winter) for weed control. The formulation was diluted at the equivalent of 75–152 L/acre at the above rates for application to give the equivalent of 4.48 or 8.96 kg ai/ha treatment.

Sampling Procedure and Preparation

Residue samples were selected from each replicate per plot about one month after the last treatment of oxadiazon. Samples (2.2 kg) from each replicate of each treated plot and 4.4 kg samples from untreated plots were taken for analysis and immediately frozen. Samples were ground while frozen, in a Buffalo® chopper, mixed thoroughly, and returned to the freezer until analyzed.

Extraction and Cleanup

Reflux 5 g subsample of dry hops (20 g green hops or hop foliage) 30 min with 200 mL 0.02N phosphoric acid. Cool sample and blend with 150 mL dichloromethane for 3 min. Transfer mixture to beaker, rinse blender bowl with small amount of water and dichloromethane, and add washings to beaker. Let mixture stand to partially separate; then filter through glass wool into separatory funnel. After dichloromethane separates, filter it through anhydrous Na_2SO_4 into 250 mL graduated cylinder. Add 1 mL concentrated HCl to water solution.

Transfer plant material back into beaker, add 50 mL dichloromethane, and stir. Then filter mixture through same funnel into separatory funnel, and shake mixture. After dichloromethane separates, filter it through Na_2SO_4 into graduated cylinder. Repeat procedure. Rinse Na_2SO_4 with dichloromethane and note total amount of solvent. On rotary evaporator, evaporate just to dryness an aliquot representing 1 g, and add 5 mL hexane (5 g aliquot was used for green hops and hop foliage). Then chromatograph sample through 20 mm od glass column containing 10 g silica gel (add anhydrous Na_2SO_4 with cotton at top and bottom of column). Wash sample onto column with 15 mL hexane and 2 mL dichloromethane. Then wash column with 85 mL hexane and 100 mL each of the following: 10, 20, 30% dichloromethane–hexane, and discard washings. Wash column with 100 mL each of 40 and 50% dichloromethane–hexane, collect and mark combined washings "A" (contains oxadiazon and methoxy metabolite residues). Wash column with 100 mL 60% dichloromethane–hexane, collect wash, and mark "B" (contains acid metabolite residue). Then wash column with 100 mL each of 70 and 80% dichloromethane–hexane and discard solvent. Wash column with 100 mL 90% dichloromethane–hexane and 200 mL dichloromethane, collect washings, and mark "C" (contains phenol metabolite residues, except for foliage samples where phenol residues are removed from column in next fraction). Wash column with 150

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This paper reports the results of research only. Mention of a pesticide in this paper does not constitute a recommendation for use by the U.S. Department of Agriculture, nor does it imply registration under FIFRA as amended. Also, mention of a commercial product in this paper does not constitute a recommendation for use by the U.S. Department of Agriculture.

Table 1. Recovery of oxadiazon and its 4 metabolites added to 3 crops

Crop	Av. rec., % (±SD)[a]				
	Oxadiazon	Methoxy	Alcohol	Acid	Phenol
Green hops (cones)	69 ± 12	78 ± 12	76 ± 15	81 ± 28	85 ± 17
Dry hops (cones)	80 ± 7	84 ± 8	80 ± 21	92 ± 21	94 ± 19
Hop foliage	65 ± 6	75 ± 14	76 ± 11	76 ± 12	71 ± 10

[a] For 5–7 determinations each.

mL 0.5% methanol–dichloromethane, collect washings, and mark "D" (contains alcohol metabolite residues, except for foliage samples which had phenol metabolite residues).

Evaporate A and C samples on rotary evaporator and dilute to specific volume with benzene for GLC determination.

Evaporate B sample as for A and C samples, add 10 mL methanol and 1 mL BF_3 solution to flask, reflux solution 30 min, and then cool. Filter solution through Ottawa sand into separatory funnel, and extract with dichloromethane. Filter solvent through anhydrous Na_2SO_4, evaporate, and dilute to specific volume with benzene for GLC determination.

Evaporate D sample as for A and C samples and add 1 mL acetone. Foliage samples require no further cleanup and, instead of adding acetone, dilute to specific volume with benzene for GLC determination. For remaining D samples, chromatograph acetone solution through 20 g Al_2O_3 acid (anhydrous Na_2SO_4 and with cotton at top and bottom, 15 mL hexane prewash). Add acetone solution to column that had 10 mL hexane on top, rinse flask with 5 mL hexane, and add rinse to column. Repeat acetone wash of flask twice, transferring washes to column. Then wash column with 150 mL hexane and 100 mL 30% acetone–hexane. Collect 150 mL 50% acetone–hexane, evaporate, and dilute to specific volume with benzene for GLC determination.

Results and Discussion

Recovery of oxadiazon and its 4 metabolites from a fortified control sample of each of the 3 crops is shown in Table 1. Equal amounts were added to the control samples, ranging from 0.25 to 2.00 ppm. The average recovery for the 3 crops for oxadiazon ranged from 64.8 to 80.0%; for the methoxy metabolite, 75.4–83.6%; for the alcohol metabolite, 75.9–79.8%; for the acid metabolite, 76.0–92.1%; for the phenol metabolite, 71.0–93.9%. Control samples representing the same amount of plant material used in recoveries and samples were adequately free of potentially interfering materials for oxadiazon and methoxy and alcohol metabolites. Control values found for the acid metabolite range from 0.573 to 1.922 ppm, and for the phenol metabolite 0.101–0.460 ppm, depending on the hop yard sample.

The residue data found in the hop leaf and cone corresponded closely with the data found by Ishizuka et al. (4) for the absorption of oxadiazon in rice plants. They found a decreasing concentration of oxadiazon in the plant parts in the order: leaves and stems > husks > hulled grains. The green hop cone did not have measurable amounts of oxadiazon residue present at

Table 2. Average total residues (ppm) of oxadiazon and its 4 metabolites in 3 crops at 2 application rates

Crop	Rate, kg ai/ha	Av. residue, ppm (±SD)[a]				
		Oxadiazon	Methoxy	Alcohol	Acid[b]	Phenol[b]
Green cones	4.48	<0.020[c] ± 0.027	0.025 ± 0.030	0.262 ± 0.470	<0.020 ± <0.020	0.224 ± 0.679
	8.96	0.125 ± 0.256	0.156 ± 0.346	1.540 ± 2.497	<0.020 ± <0.020	0.268 ± 0.274
Dry cones	4.48	<0.100 ± 0.122	ND[d]	<0.100 ± 0.103	<0.020 ± <0.020	0.211 ± 0.649
	8.96	0.305 ± 0.133	<0.100 ± 0.065	ND	0.879 ± 0.901	0.100 ± 0.124
Hop foliage	4.48	0.058 ± 0.031	0.056 ± 0.040	0.021 ± 0.056	<0.020 ± <0.020	<0.020 ± <0.020
	8.96	0.163 ± 0.173	0.046 ± 0.023	0.061 ± 0.150	<0.020 ± <0.020	<0.020 ± <0.020

[a] Results have been corrected for average recoveries; 5–7 determinations.
[b] Results have been corrected for average check values found.
[c] Below the minimum sensitivity of the method: 0.1 ng for each.
[d] None detectable.

the recommended treatment rate of 4.48 kg ai/ha; however, when this rate was doubled, the oxadiazon residue averaged 0.13 ppm (Table 2). Measurable residues were found for each of the metabolites in the green cones.

Drying the cone concentrated the residue of oxadiazon, but apparently produced a loss of the metabolite residues. The methoxy and alcohol metabolite residues in the dry cone either were not measurable or were below the minimum sensitivity of the method.

Bingham et al. (6) analyzed peanut plants and found about 1 ppm oxadiazon equivalent in peanut hulls 131 days after treatment. A large concentration of residue equivalents was not found in any of the hop samples when they were sampled one month after the last treatment.

Guardigli (5) used a number of cleanup methods to remove plant material for residue analysis. None of these methods were satisfactory in removing the hop resins from the samples. We used column chromatography through silica gel to clean samples. This method had the advantage of removing the hop resins and also of separating the metabolites, which facilitated later gas chromatographic analysis.

Acknowledgment

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References

(1) Ambrosi, D., Isensee, A. A., & Macchia, J. A. (1978) *J. Agric. Food Chem.* **26,** 50–53
(2) Ambrosi, D., Kearney, P. C., & Macchia, J. A. (1977) *J. Agric. Food Chem.* **25,** 868–872
(3) Hirata, H., & Ishizuka, K. (1975) *Agric. Biol. Chem.* **39,** 1447–1454
(4) Ishizuka, K., Hirata, H., & Fukunara, K. (1975) *Agric. Biol. Chem.* **39,** 1431–1446
(5) Guardigli, A. (1978) *Rhodia Analytical Method No.* 150, 1–26
(6) Bingham, S. W., Shaver, R. L., & Guyton, C. L. (1980) *J. Agric. Food Chem.* **28,** 735–740

Spectrophotometric Determination of Carbaryl in Grains

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A method has been developed for determining carbaryl (1-naphthyl *N*-methyl carbamate) in grains, based on hydrolysis of carbaryl with methanolic potassium hydroxide to 1-naphthol, reaction with 4-aminophenazone in the presence of alkaline oxidizing agent, and spectrophotometric measurement at the absorption maximum at 475 nm. The relationship between absorbance and concentration is linear in the range of 0.5-20 μg/mL. The method can be applied to levels as low as 0.3 ppm carbaryl in grains.

Carbaryl, 1-naphthyl *N*-methyl carbamate, is a contact insecticide with slight systemic activity. It is used in pest control in India and many tropical countries and thus the determination of its residues has become imperative. Miskus et al. (1) and Johnson (2) reported that alkaline hydrolysis of carbaryl yields 1-naphthol which, when coupled with *p*-nitrobenzene diazonium fluoborate, produces a color with an absorption maximum at 590 nm in alkaline medium. The reaction is sensitive to 5 μg. The colorimetric method of Vonesch and de Riveros (3) is based on the reaction of the insecticide with diazotized 2,5-dichloroaniline. This reaction is sensitive to 0.1 μg with a detection limit of 0.2 ppm. Another spectrophotometric method based on the reaction of carbaryl with diazotized *o*-toluidine has also been reported (4). The resultant red product is measured at 520 nm with a sensitivity of 0.1 μg/20 g sample. Klisenko (5) developed a colorimetric method based on coupling carbaryl with diazotized sulfanilic acid and measuring the dye at 500 nm. This reaction is sensitive to 1 μg carbaryl/2.5 mL.

We developed a sensitive spectrophotometric method based on the reaction of 1-naphthol, the hydrolysis product of carbaryl, with 4-aminophenazone in the presence of an alkaline oxidizing agent. The occasional unavailability of other reagents has necessitated a search for an alternative chromogen.

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METHOD

Apparatus and Reagents

(a) *Chromatographic column.*—400 × 20 mm id Pyrex glass column.

(b) *Spectrophotometer.*—Perkin-Elmer Model 475 with 1 cm silica cell.

(c) *Blender.*—Fire and explosion proof (Russell & Stall Waring Products Division, Dynamics Corp. of America, New Hartford, CT).

(d) *Carbaryl.*—Reference standard material (Union Carbide Corp., Chemicals Div., Technical Center, South Charleston, WV). Prepare 100 ppm working standard solution (100 μg/mL) of carbaryl in methanol.

(e) *4-Aminophenazone.*—Analytical reagent grade (Koch-Light Laboratories Ltd., Coinbrook, Bucks., UK). Prepare 3% solution in water.

(f) *Potassium ferricyanide.*—Analytical reagent grade (British Drug Houses, London, UK). Prepare 20% in water.

(g) *Hydrochloric acid.*—35.4%, sp. gr. 1.18. Dilute to 0.2N with water.

(h) *Ammonium hydroxide.*—25% NH_3, sp. gr. 0.91. Analytical reagent grade (British Drug Houses). Dilute 1:2 with water.

(i) *Methanolic potassium hydroxide.*—0.2N in methanol.

(j) *Carbaryl formulation.*—50% wettable powder (Union Carbide Co., Bhopal, India).

(k) *Silica gel.*—60-120 mesh, column chromatography grade (British Drug Houses). Activate 3 h at 105°C.

(l) *Sodium sulfate.*—Anhydrous (British Drug Houses).

Preparation of Standard Curve

Pipet 0.0, 0.25, 0.50, 1.0, 2.5, 5.0, 7.5, and 10.0 mL aliquots of carbaryl working standard solution (25-1000 μg) into clean, dry test tubes. Add 2.5 mL 0.2N methanolic KOH to each tube and shake 5 min. Neutralize alkali by adding 0.2N HCl and adjust pH to 9-10 with 1:2 NH_4OH. Add ca 2 mL 3% 4-aminophenazone to each tube followed by 2 mL 20% $K_3Fe(CN)_6$. Orange dye with absorption maximum at 475 nm (Figure 1) is stable >8 h. Quantitatively transfer solutions to clean, dry separatory funnels. Let stand 5 min

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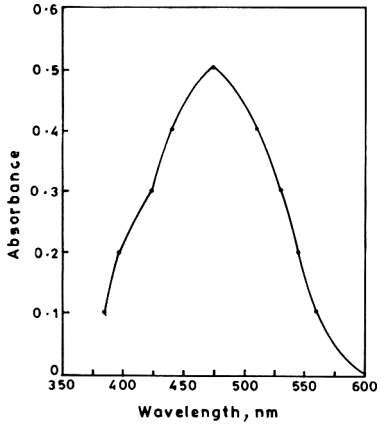

Figure 1. Absorption spectrum of antipyrine dye.

for full color development and extract orange dye with three 10 mL portions of $CHCl_3$. Pass $CHCl_3$ extract through anhydrous Na_2SO_4 and wash adsorbed color from Na_2SO_4 3 times with 5 mL portions of $CHCl_3$. Collect $CHCl_3$ extract in 50 mL volumetric flask and dilute to volume with $CHCl_3$. Read absorbance of orange dye at 475 nm in spectrophotometer against blank similarly prepared. Plot of concentration of carbaryl (0.5–20 μg/mL) vs absorbance will yield straight line.

Determination of Recovery

Transfer 100 g grain (rice, wheat, and jowar) and pulse (pigeon pea, black gram, and green gram) samples to Waring blender and blend 5 min with 200 mL $CHCl_3$. Spike samples in blender with 30, 60, 120, and 240 μg carbaryl in 5 mL methanol. Blend spiked samples 2 min. Decant $CHCl_3$ into 250 mL Erlenmeyer flask through Whatman No. 1 filter paper and retain residue in blender. Repeat blending and filtering twice with 100 mL portions of $CHCl_3$ and transfer residue completely onto filter paper. Rinse blender with 20 mL $CHCl_3$ and add rinse to filter paper. Wash residue on filter paper twice with 20 mL portions of $CHCl_3$. Combine $CHCl_3$ extracts and evaporate to 10 mL in rotary vacuum evaporator. Plug bottom of chromatographic column with glass wool. Pack column with 10 g silica gel and add Na_2SO_4 to 2 cm height above silica gel. Prewash column with 100 mL $CHCl_3$ and discard eluate. Pour $CHCl_3$ extract (concentrated) onto column and elute insecticide with 200 mL $CHCl_3$ at 5 mL/min. Collect eluate in 500 mL Erlenmeyer flask. Remove solvent in rotary vacuum evaporator and dissolve residue in 10 mL methanol. Develop color as described under preparation of standard curve and measure absorbance against crop control (blank).

Table 1. Recovery of carbaryl from spiked grain and pulse samples

Sample	Added, μg	Found, μg[a]	Rec., %
Rice (polished)	30	29.97 ± 0.33	99.9
	60	58.98 ± 0.40	98.3
	120	117.36 ± 0.57	97.8
	240	232.80 ± 1.37	97.0
Wheat	30	30.00 ± 0.00	100.0
	60	59.46 ± 0.14	99.1
	120	117.48 ± 1.05	97.9
	240	233.04 ± 2.45	97.1
Jowar (sorghum)	30	29.70 ± 0.24	99.0
	60	59.16 ± 0.11	98.6
	120	115.56 ± 0.72	96.3
	240	225.60 ± 0.66	94.0
Pigeon pea	30	29.79 ± 0.71	99.3
	60	59.40 ± 0.74	99.0
	120	115.56 ± 0.51	96.3
	240	228.00 ± 1.70	95.0
Black gram	30	29.76 ± 0.35	99.2
	60	58.86 ± 1.89	98.1
	120	115.20 ± 1.44	96.0
	240	227.52 ± 1.79	94.8
Green gram	30	29.67 ± 0.40	98.9
	60	58.68 ± 1.31	97.8
	120	115.56 ± 0.56	96.3
	240	226.80 ± 1.22	94.5

[a] Average ± standard deviation of 8 analyses.

Table 2. Analysis of 50% wettable powder formulation of carbaryl

Batch	Wt of sample, mg	Carbaryl found, mg	Carbaryl, %
1	1.120	0.543	48.50
	1.810	0.873	48.20
	2.360	1.121	47.50
	3.000	1.410	47.00
			Av. 47.50
2	0.760	0.372	49.03
	1.950	0.950	48.71
	2.200	1.060	48.18
	3.200	1.492	46.65
			Av. 48.14
3	0.950	0.463	48.75
	1.660	0.800	48.25
	3.320	1.581	47.62
	4.000	1.875	46.85
			Av. 47.87

Formulation Analysis

Weigh 0.760–4.0 mg 50% carbaryl and dissolve in 10 mL methanol in centrifuge tubes. Centrifuge 5 min at 5000 rpm and decant supernate into clean, dry 25 mL volumetric flask. Re-extract residue in tubes with another 10 mL portion of methanol as before and combine extracts. Dilute to 25 mL with methanol and use known aliquots of solution for color development as outlined in standard curve preparation.

Results and Discussion

To check recovery of carbaryl by method described, grain and pulse samples were spiked with known amounts of carbaryl and analyzed. Recoveries of carbaryl from grains (rice, wheat, jowar) and pulses (pigeon pea, black gram, and green gram) varied from 94 to 100% (Table 1). The described spectrophotometric method is sensitive to 0.5 μg with a detection limit of 0.3 ppm, which makes it useful for determining insecticides in microquantities. Determination of carbaryl in 50% wettable formulation is given in Table 2. Average carbaryl content in 3 batches of the formulation varied from 47.50 to 48.14% against the declared value of 50%.

The linear relationship between the absorbance at 475 nm and the concentration of carbaryl is valid up to 20 μg/mL reaction mixture. Beyond this level, the relationship is not linear and recoveries are inconsistent. Although maximum color development takes place in 5 min and color is stable for more than 8 h, absorbance should be recorded within 1 h because of cloud formation. A similar problem is encountered when the orange dye extracted with chloroform is not passed through anhydrous sodium sulfate.

The proposed colorimetric method is more sensitive than the colorimetric method (1) that specifies fluoborate as a chromogenic salt. Carbaryl can be estimated at levels as low as 0.5 μg as opposed to 5 μg by the latter method. Although the present method is not as sensitive as other colorimetric methods (2–5) with reported sensitivity of 0.1–0.4 μg, the method is very useful for the determination of carbaryl from grains (permissible limit 2.5–5.0 ppm). 4-Aminophenazone is a common reagent used in the laboratories for the determination of phenols and as such it will serve as an alternative sensitive reagent for the determination of carbaryl from grains and formulations.

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References

(1) Miskus, R., Gordon, H. T., & George, D. A. (1959) *J. Agric. Food Chem.* **7**, 613–614
(2) Johnson, D. P. (1964) *J. Assoc. Off. Agric. Chem.* **47**, 283–286
(3) Vonesch, E. E., & de Riveros, M. H. C. K. (1971) *J. Assoc. Off. Anal. Chem.* **54**, 128–130
(4) Rangaswamy, J. R., & Majumder, S. K. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 592–594
(5) Klisenko, M. A. (1965) *Zh. Anal. Khim.* **20**, 634–636

Derivatization and Cleanup Improvements in Determination of Residues of Glyphosate and Aminomethylphosphonic Acid in Blueberries

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A new method based on development of the 2-chloroethyl *N*-heptafluorobutyryl derivatives of glyphosate and its major metabolite aminomethylphosphonic acid is reported for fortified blueberry samples. The concentrated aqueous extract is chromatographed on a gel permeation column in a low pH eluant. Under these conditions, sugar is eliminated by permeation and pigments are strongly adsorbed. Additional sugar is eliminated with a small cation exchange column and then the sample is easily dried and derivatized. The derivatized compounds are extracted from an aqueous environment with hexane and analyzed by gas chromatography with electron capture detection. This procedure requires significantly less sample handling time and provides greater sensitivity for glyphosate than the commonly used procedure recommended by EPA.

Glyphosate (*N*-(phosphonomethyl) glycine) is the active ingredient of the broad spectrum, non-selective herbicide Roundup (a product of the Monsanto Co.). This compound is effective in the control of a wide range of weeds (1–11) and at the same time is relatively non-toxic to mammals. The LD_{50} based on oral feeding of male rats is 4320 mg/kg (12). Properties which make this compound effective include high water solubility, rapid absorption and translocation by plants, and low degrees of in vivo metabolism and degradation. A recent review calls glyphosphate a unique compound in that it is the only registered herbicide which acts by disruption of phenolic metabolism (13). The above factors have led to the development of glyphosate as a very important herbicide.

Because of its significance, a sensitive, accurate, widely applicable crop residue analysis for glyphosate and its major metabolite aminomethylphosphonic acid (AMPA) (14) is essential. Special complexities arise in the quantitation of these compounds because they are extracted in water and are insoluble in organic solvents. Cleanup and conversion to volatile molecules for analysis has proven difficult for these compounds in an aqueous mixture. The literature cites several methods concerning the determination of the metabolite and/or the pesticide including: fluorogenic HPLC analysis (15); thin layer chromatography (16–18); amino acid analyzer detection of the ninhydrin-glyphosate reaction product (19); direct analysis by HPLC (20); and gas chromatography (21). The method developed by the manufacturer (Monsanto) and recommended by the Environmental Protection Agency (22) involves water extraction and elimination of pigments by charcoal treatment and organic solvent partitioning. This is followed by anion exchange chromatography, cation exchange chromatography, acylation, and methylation. The derivatized products are analyzed by phosphorus mode flame photometric gas chromatography. This procedure has been adapted for use in this laboratory (23) for numerous analyses on a large variety of crops and generally is time consuming, involves large residue losses, and results in many chromatographic interferences. In this study, gel filtration cleanup and new derivatives are investigated as improvements in this procedure for the determination of glyphosate and aminomethylphosphonic acid residues in blueberries.

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METHOD

Reagents

(a) *Solvents.*—Pesticide grade ethyl acetate and hexane (Mallinckrodt, Inc., St. Louis, MO); Aquasol-2 (New England Nuclear, Boston, MA).

(b) *Derivatization reagents.*—Heptafluorobutyric anhydride (Pierce Chemical Co., Rockford, IL); BCl_3–2-chloroethanol (10%) (Applied Science Laboratories, PO Box 440, State College, PA 16801).

(c) *Sodium sulfate.*—Anhydrous (Mallinckrodt, Inc.).

(d) *Standards.*—Glyphosate, ^{14}C-glyphosate, and ^{14}C-aminomethylphosphonic acid (gifts from Monsanto Co.); unlabeled aminomethylphosphonic acid (Sigma Chemical Co., PO Box

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1450, St. Louis, MO).

(e) *Standard solutions.*—Stock solutions of 200 μg glyphosate and aminomethylphosphonic acid/mL water. Dilute as needed.

Apparatus

(a) *Gas chromatograph.*—Automatically integrating, microprocessor-controlled Hewlett-Packard Model 5840A gas-liquid chromatograph equipped with ^{63}Ni electron capture detector and 327 cm × 4 mm id glass column packed with 10% DC-200 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories). Parameters: argon-methane (95 + 5) carrier gas flow 60 mL/min; injection volume 5 μL; detector 300°C; injection port 250°C; column oven— initial 130°C, hold 15 min, program sequentially at 30°/min to 210°C, hold 7 min, and then 220°C for 13 min.

(b) *Gel filtration apparatus.*—Pharmacia K 26/100 column (2.6 cm × 100 cm) packed with Bio-Gel P-2 (Bio-Rad Laboratories, 32nd & Griffin, Richmond, CA) and equipped with Pharmacia 5A-50 sample applicator. Gilson Minipuls 2 peristaltic pump maintains system flow rate at 3 mL/min.

(c) *Liquid scintillation counter.*—Searle Analytic Model 92.

(d) *Strong cation exchange column.*—2.2 × 6.5 cm AG 50W-X8 (Bio-Rad Laboratories).

Calibration of Gel Permeation Column

Fortify 21 g blueberry sample with 15 μg each of glyphosate and aminomethylphosphonic acid. Add 125 000 cpm of ^{14}C-glyphosate and 70 000 cpm of ^{14}C-aminomethylphosphonic acid. Treat sample according to sample preparation section and apply to gel permeation column equilibrated in pH 2.1 water. Elute at 3 mL/min with pH 2.1 water and collect 190 mL void followed by thirty 8 mL fractions. Monitor 1 mL of each fraction for radioactive content by counting 20 min in 10 mL Aquasol-2 in scintillation counter. Plot cpm vs fraction number.

Calibration of Strong Cation Exchange Column

Equilibrate AG 50W-X8 column in pH 2.1 water. Apply blueberry sample which has been cleaned up on Bio-Gel P-2 column and fortified with 15 μg unlabeled pesticide and metabolite as well as 2.1 μg radioactive standard. Collect 5 mL samples until 21 mL eluant has been collected; change eluant to pH 7.0 deionized water. Monitor each fraction by counting 1 mL sample in 10 mL Aquasol-2 in scintillation counter.

Sample Preparation

Fortify representative 25 g blueberry samples to various levels with both glyphosate and aminomethylphosphonic acid. Homogenize and sonicate each twice in 100 mL water for 5 min with Polytron apparatus. Centrifuge 10 min at 7000 × g after each homogenization and combine supernates in flash evaporator flask after passage through glass wool plug. Add 2 drops of 30% NaOH until pigments turn brown. Concentrate to ca 50 mL by rotary evaporation. Extract each sample 3 times with 100 mL ethyl acetate by adding stirring bar and solvent to flask, stirring for 3 min, and aspirating ethyl acetate. Concentrate to 16 mL with rotary evaporation and adjust pH to 2.1 with 6N HCl. Pass sample through Whatman No. 1 paper in a syringe-loaded apparatus. Apply 10 mL (representing 15 g sample) to Bio-Gel P-2 column through sample applicator. Elute at 3 mL/min with water adjusted to pH 2.1 with HCl. Discard first 290 mL and collect next 100 mL. Concentrate collected eluates by rotary evaporation to 4 mL and apply this sample to AG 50W-X8 cation exchange column (2.2 × 6.5 cm) equilibrated in pH 2.1 water. Discard 21 mL of pH 2.1 water eluate (including application volume), change eluant to deionized water which has been adjusted to pH 7.0 with dilute NaOH, and collect next 150 mL. Reduce 150 mL volume to 3–4 mL with vacuum rotary evaporator in 50°C water bath. Transfer to reaction tube and take to dryness under nitrogen in water bath at ca 80°C. Store these samples in evacuated desiccator containing phosphorus pentoxide.

Derivatization of Samples

Add 1.5 mL BCl_3–2-chloroethanol to each tube, seal tube, and heat 45 min at 110°C. Evaporate excess reagent in boiling water bath under nitrogen for 30 min. Add 100 μL heptafluorobutyric anhydride to each tube, seal, and heat to 110°C for 45 min. Add 1 mL water to each tube; then add 1 mL 5% aqueous ammonia solution. Extract aqueous phase 3 times with 4 mL hexane and pass hexane through small bed of anhydrous Na_2SO_4. Concentrate combined extracts to 1 mL under nitrogen flow and analyze by electron capture GLC. Prepare 7-point standard curve ranging from 0.005 to 15 μg/mL, according to this procedure. Prepare standards each time an analysis is conducted.

Results and Discussion

Blueberries have proven to be a particularly difficult crop to analyze because of large amounts

of extractable sugars and pigments. The bulk concentrations of these constituents make the samples impossible to manipulate in small volumes. Gelling of the crop extract after volume reduction further complicates the procedure and, in addition to these difficulties, water extraction followed by ion exchange cleanup tends to isolate phosphorus-containing compounds (peptides, sugars, nucleic acid, etc.) with functional groups ionically similar to glyphosate and its metabolite. These problems have accentuated the normal problems with this residue analysis.

Gelling of the crop extract can be prevented by adjusting the sample to pH ≥8.0 (24, 25). This can be accomplished without measurement in the extracts by adding sodium hydroxide until the crop pigments turn brown. Subsequently, the volume is reduced and the sample is extracted with ethyl acetate to remove sufficient amounts of sugars and/or pectic substances which will allow the pH of the aqueous pesticide-containing fraction to be decreased without gel formation. Aqueous gel permeation chromatography has been successfully used for the chromatography of mixtures of oligosaccharides (26–28) and such applications suggest it might be useful for the partial purification of glyphosate and its metabolite from a concentrated sugar solution. Polyacrylamide gel filtration media have also been shown to absorb polymerized anthocyanins (the major pigment in blueberries) under acidic conditions (29). Chromatography of the blueberry extracts on Bio-Gel P-2 (a neutral, hydrophilic, polyacrylamide gel, exclusion limit 1800 daltons) performs 2 major functions: elimination of sugars by molecular sieving, and elimination of pigments by adsorption. Radioactivity in the spiked crop sample applied to the gel column is found in the fractions representing 202–256 mL. Approximately 85% of the counts applied to the column were recovered. Compounds of similar charge but greater molecular weight will be separated from glyphosate and aminomethylphosphonic acid at this point. This step circumvents the total reliance on ion exchange chromatography.

Enough sugar remains to prevent complete drying of the sample, so further cleanup is necessary before derivatization. Simple batch processing on AG 50W-X8 cation exchange resin will eliminate a major portion of the remaining sugar. Visual examination of schlieren lines in the eluate as it drips from the column indicates that the sugars elute in the first 21 mL. The mode of separation appears to be retardation rather than ion exchange because both compounds (glyphosate first at 28 mL) will elute from the column without a change in eluant, although the use of pH 7.0 water accelerates the elution of both compounds. The sample can be easily taken to dryness and derivatized following the cation exchange column. The established procedure calls for a complete separation of glyphosate and its metabolite on AG 50W-X8 resin utilizing radioactive tracer monitoring of collected fractions. This step follows processing of the sample in a 2000 mL volume on an anion exchange column and precedes a 2-step derivatization requiring acylation with trichloroacetic acid and methylation with fresh ether-distilled diazomethane. The aqueous gel permeation, batch cation exchange, and derivatization modifications suggested in this study significantly reduce analysis time by providing for the utilization of smaller sample-handling volumes and commercially available, ready-to-use acylation and alkylation reagents.

Lower limits of detection and higher recovery data have been achieved with this method than have been previously experienced. A 5-point standard curve (μg/mL vs integrated area) shows a 0.993 linear coefficient of determination for glyphosate and a 0.996 coefficient for aminomethylphosphonic acid. All peaks were quantitated by tangent skim mediated integration and expressed as unit areas. The minimum level of detectability for standards is 15 pg injected for the pesticide and 25 pg injected for the metabolite. The integrated area of these peaks is well above background and consistent through repeat analyses. Peaks can be obtained, which are 2 times background for lower concentrations of standards, but they are not consistently integrated, and therefore are not used for quantitative purposes. The EPA-approved method, as applied in this laboratory, achieves minimum detectability for both compounds with 16 700 pg injected and flame photometric gas chromatography. Recoveries for fortified 15 g blueberry samples (av. ± SD are for duplicates) are 94% (1 ppm), 61% (0.5 ppm), 66 ± 1% (0.05 ppm), and 52 ± 9% (0.01 ppm) for glyphosate and 108% (1 ppm), 108% (0.5 ppm), and 102 ± 0.5% (0.05 ppm) for aminomethylphosphonic acid. All values reported for both standards and fortified crops are for the molecular weight equivalents of the underivatized compounds. Figure 1 shows representative chromatograms for a 12.5 μg standard, 0.5 ppm crop spike, and a control crop. All samples have equal amounts of both pesticide and metabolite. Interference with the chroma-

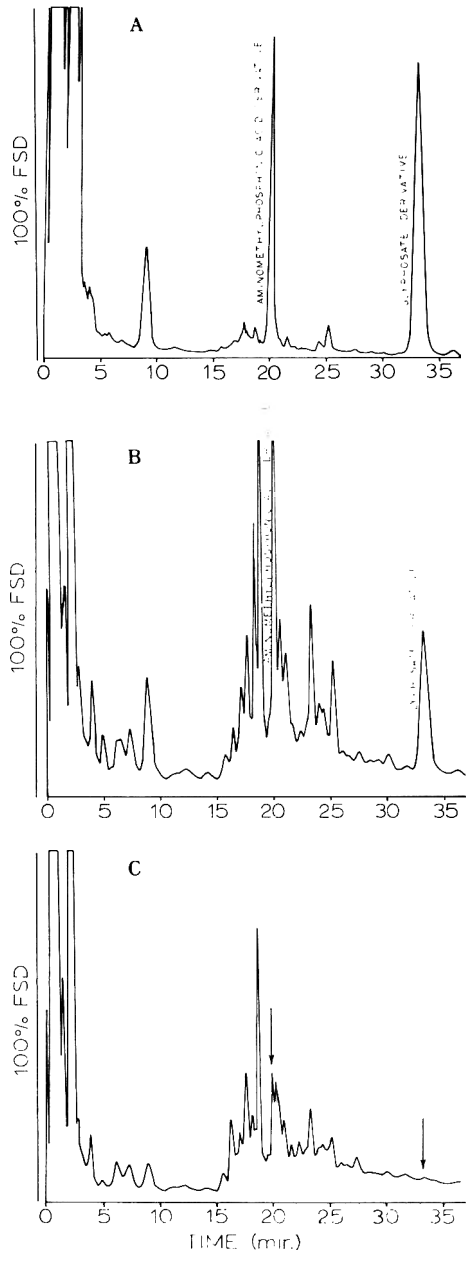

Figure 1. Chromatograms (attenuation 2^{14}) of A, derivatized standard (5 ng injected) in both glyphosate and metabolite; B, 0.5 ppm spiked crop sample (containing both compounds) following cleanup and derivatization; and C, control chromatogram showing minor interferences (marked by arrows).

Retention times are 20.19 min for derivatized metabolite and 33.27 for derivatized glyphosate. Scale of these chromatograms has been expanded to accentuate background, illustrate lack of interferences with glyphosate, and show interference which causes quantitation difficulties with 0.01-ppm fortification level of aminomethylphosphonic acid.

tography of glyphosate is minimal as illustrated by the control chromatogram. The integrated area of this interference ranged from its usual value of zero to a maximum of half the integrated area of the 0.01 ppm fortification level. Interferences with the chromatography of the derivatized aminomethylphosphonic acid (about 0.018 ppm) were greater than those found for glyphosate. As a result, recovery of the 0.01 ppm fortification level is not reported because the integrated peaks were not consistently distinguishable from background. The peaks from the 0.05 ppm fortification level were easily quantitated and were always greater than 2 times background. The EPA method as applied in this laboratory provides a minimum quantitatable recovery for 50 g blueberry samples fortified at the 0.025 ppm level. Obtaining a 0.05 ppm minimum recovery for the metabolite rather than 0.025 ppm is a small sacrifice for the analysis time and difficulty saved by using 15 g crop samples. A larger crop sample would probably provide lower levels of sensitivity, but it would also cause less efficient utilization of the gel permeation cleanup. Five 15 g crop samples can be chromatographed on a Bio-Gel P-2 column before the pigments must be removed with a 4M urea solution adjusted to pH 8.0 with sodium hydroxide. It is a simple matter to re-equilibrate the column in pH 2.1 water and use it for more samples. Processing samples through the column in this manner (especially if the gel permeation system is automated) is considerably faster than the anion exchange step of the EPA procedure and requires little analyst attention. The use of larger crop sizes to obtain a higher level of recovery for the metabolite would require that pigments be removed more frequently and a subsequent reduction in sample processing efficiency would be experienced. It is also important to note that utilizing this new method (with 15 g samples) provides a minimum recovery of glyphosate (0.01 ppm) which is 5 times greater than that of the EPA method (0.05 ppm) which uses 50 g samples and flame photometric detection. This could be significant in the actual detection of glyphosate in field-treated blueberry samples because residues have not yet been reported from pre-emergence or post-emergence studies.

Mass spectral studies have verified the structures of these new derivatives (30). Both aminomethylphosphonic acid and glyphosate are amino-acylated and all hydroxyls are 2-chloroethylated. These 2-chloroethyl-*N*-heptafluorobutyryl derivatives are extremely sensitive to

electron capture detection, but offer versatility in that they can also be quantitated by flame photometric and nitrogen-phosphorus detection. Samples analyzed by these element-specific detectors usually require less cleanup than those analyzed by electron capture detection. The extraction of these derivatives from an aqueous environment with hexane is also a significant cleanup step. By comparison ethyl acetate extraction of the derivatives results in unresolvable interferences with electron capture detection. The element-specific detection and solvent-partitioning advantages could lead to the elimination of the gel permeation and/or the ion exchange cleanup steps for crops with fewer pigments and sugars than blueberries. Two additional advantages of these derivatives are the use of commercially purchased 2-chloroethanol rather than fresh ether-distilled diazomethane, and the stability of the derivatives (undiminished response 3 weeks after derivatization).

Conclusion

The method presented in this paper for the determination of glyphosate and aminomethylphosphonic acid in blueberries provides potential for a significant reduction in the analysis time required to process large numbers of samples and an increased sensitivity for glyphosate.

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References

(1) Schultz, M. E., & Burnside, O. C. (1979) *Agron. J.* **71,** 723–730
(2) Schultz, M. E., & Burnside, O. C. (1980) *Weed Sci.* **28,** 13–20
(3) Wyrill, J. B., & Burnside, O. C. (1976) *Weed Sci.* **24,** 557–566
(4) Cornell, J. T., & Jeffery, L. S. (1977) *Proc. South. Weed Sci. Soc.* **30,** 75–80
(5) McWhorter, C. G. (1977) *Weed Sci.* **25,** 135–141
(6) Parochetti, J. V., Wilson, H. P., & Burt, G. W. (1975) *Weed Sci.* **23,** 395–400
(7) Richardson, J. T., & Trans, R. E. (1977) *Proc. South. Weed Sci. Soc.* 30, 37
(8) McWhorter, C. G., Jordan, T. N., & Wills, G. D. (1980) *Weed Sci.* **28,** 113–118
(9) Gottrup, O. P. A., O'Sullivan, P. A., Schraa, R. J., & Vander, W. H. (1976) *Weed Res.* **16,** 197–201
(10) Rioux, R., Bandeen, J. P., & Anderson, G. W. (1974) *Can. J. Plant Sci.* **54,** 397–401
(11) Suwanketnikom, R., & Penner, D. (1976) *Proc. North Cent. Weed Control Conf.* **31, 141**
(12) Watts, R. R. (1980) *Analytical Reference Standards and Supplemental Data for Pesticides and Other Organic Compounds,* Environmental Protection Agency, Environmental Toxicology Division, Research Triangle Park, NC, p. 52
(13) Hoagland, R. E., & Duke, S. O. (1981) *Weeds Today* (Late Spring), 21–23
(14) Rueppel, M. L., Brightwell, B. B., Schaefer, J., & Marvel, J. T. (1977) *J. Agric. Food Chem.* **25,** 517–528
(15) Moye, H. A., & St. John, P. A. (1980) *Pesticide Analytical Methodology,* ACS Symp. Ser., No. 136, American Chemical Society, Washington, DC, pp. 89–102
(16) Young, J. C., Kahn, S. U., & Marriage, P. B. (1977) *J. Agric. Food Chem.* **25,** 918–922
(17) Ragab, M. T. H. (1978) *Chemosphere* 7, 143–153
(18) Sprankle, P., Sandberg, C. L., Meggitt, W. F., & Penner, D. (1978) *Weed Sci.* **26,** 673–674
(19) Ekstrom, G., & Johansson, S. (1975) *Bull. Environ. Contam. Toxicol.* **14,** 295
(20) Burns, A. J., & Tomkins, D. F. (1979) *J. Chromatogr. Sci.* **17,** 333–335
(21) Tate, R. L., & Alexander, M. (1974) *Soil Sci.* **118,** 317
(22) Food and Drug Administration (1977) *Pesticide Analytical Manual,* Vol. II (1977) Transmittal No. 77-3
(23) Thompson, N. P., Lynch, A. A., & Bardalaye, P. C. (1980) *Proc. Fla. State Hort. Soc.* **93,** 159–160
(24) Charley, H. (1970) *Food Science,* The Ronald Press Co., New York, NY, pp. 487–500
(25) Kobata, A. (1972) *Methods Enzymol.* **28,** 262–271
(26) Kobata, A. (1978) *Methods Enzymol.* **50,** 216–235
(27) Tokunaga, M., Endo, M., & Yosizawa, Z. (1979) *Tohoku J. Exp. Med.* **127,** 17–24
(28) John, M., Trenel, C., & Dellweg, H. (1969) *J. Chromatogr.* **42,** 476–484
(29) Lin, R. I., & Hilton, B. W. (1980) *J. Food Sci.* **45,** 297–306
(30) Guinivan, R. A., Thompson, N. P., & Wheeler, W. B. (1982) *J. Agric. Food Chem.* (submitted)

Simple Colorimetric Method for Determination of Organothiophosphate Insecticides in Technical Materials and Formulations

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A simple colorimetric method based on the reaction of benzophenone with thiophosphate is described. The method is specific for compounds containing the P=S moiety, gives recoveries of 99 ± 1%, and obeys Beer's law for the concentration range 35-350 μg/mL. Other sulfur-containing compounds such as sulfa drugs, thiobarbiturate, thiourea, carbon disulfide, and dithiocarbamates do not interfere in the reaction. The method is useful in studying the shelf-life of formulations.

Various methods have been reported for determining organothiophosphate insecticides, most of which are based on reactions with the phosphoric acid moiety. Getz and Watts (1) reported a rapid colorimetric method for determining organophosphate pesticides by reaction with 4-(*p*-nitrobenzyl)pyridine in a slightly alkaline solution at 175-180°C. Turner (2) further modified the method and carried out the reaction at a slightly lower temperature, 100°C. The color is very unstable and stability varies according to pesticides; erratic results occur due to rapid evaporation of solvents (2). Ott and Gunther (3) reported a method for determining organothiophosphates by wet digestion oxidation or alkali hydrolysis followed by treatment with ammonium molybdate and 1-amino-2-naphthol-4-sulfonate to form a molybdenum blue complex.

Certain methods are useful for individual organophosphorus pesticides. A method involving formation of copper complex has been reported for malathion (4-7). Enos and Frear (8) reported a method for dimethioate based on formation of methylene blue. George et al. (9) formed a complex between dimethoate and 1-chloro-2,4-dinitrobenzene. Recently, a method based on reaction of mercurous nitrate with *p*-phenyl organophosphate insecticides has been reported (10). Several workers used an enzymic method of cholinesterase inhibition (11). Such esterase-activity measurements are useful only as screening tests, provided negative results are obtained. Previous identification of the pesticide is essential, because cholinesterase is inhibited by many compounds: organochlorines (12), barbiturates (13, 14), carbamates (15), and chloral hydrate (13).

A number of other methods based on techniques such as paper chromatography (16), thin layer chromatography (17), gas-liquid chromatography (18), high pressure liquid chromatography (19), and gas chromatography-mass spectrometry (20, 21) are used for quantitative determination of such insecticides.

We describe a simple colorimetric method for determining organothiophosphate insecticides, based on the reaction of the P=S moiety. The blue reaction product, thiobenzophenone (22), has an absorption maximum at 598.8 nm (Figure 1).

Experimental

Use analytical grade reagents that are free of moisture.

Preparation of Sample

Accurately weigh 10 g material. Extract 5 times with 100 mL portions of ethyl ether. Collect all extracts and evaporate to near dryness under vacuum at ≤30°C. Dissolve residue in ca 70 mL acetone and dilute with acetone to 100 mL in volumetric flask. Dilute this stock solution appropriately with acetone to concentration range of 100-1000 μg/mL.

Dilution will depend on original concentration of formulations, e.g., dilute Cythion stock solution (Synamide India, 10 g/100 mL), which is 50% malathion, 1:100 for a sample concentration of 500 μg/mL; dilute Dalf stock solution (Bayer India, 10 g/100 mL), which is 2% fenthion, 1:4 for sample concentration of 500 μg/mL.

Procedure

Place 1 g accurately weighed benzophenone in test tube with 3 mL graduation mark. Heat tube on microflame until benzophenone melts completely. Add 1 mL sample solution in acetone. Heat contents of tube and boil 4 min. Cool tube to room temperature and dilute contents to 3 mL mark with methanol. Measure

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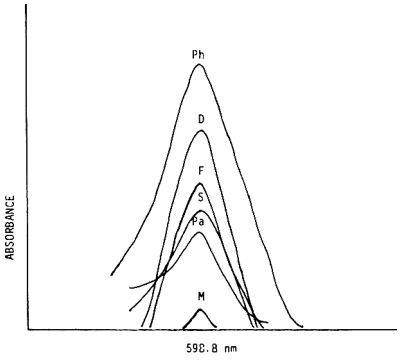

Figure 1. Absorption maximum of reaction product: M, malathion; Pa, parathion; S, sumithion; F, fenthion; D, dimethoate; Ph, phorate.

absorbance of blue reaction product at 599 nm against reagent blank in 1 cm cell. For reagent blank, melt 1 g benzophenone in test tube, cool to room temperature, and dilute to 3 mL mark with methanol.

Calibration Graph

Prepare series of standards of thiophosphate authentic samples by diluting stock standard solutions appropriately to give concentrations of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 μg/mL. Measure color produced for each standard by above procedure. Plot of absorbance against concentration of thiophosphate gives straight line passing through origin and obeys Beer's law for concentration ranges for different organothiophosphates shown in Table 1.

$$\text{g Insecticide/100 g sample} = (A/A') \times (C'/D) \times 100$$

where A and A' = absorbance of sample and standard, respectively; C' = concentration of standard, mg/g; D = dilution of sample.

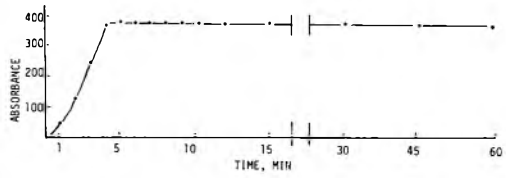

Figure 2. Graph showing maximum heating time and stability of color.

Results and Discussion

The effect of time of heating and stability of color formed was checked with 50 μg of each insecticide. The reaction is complete after 4 min as shown in Figure 2. Organothiophosphates such as malathion, parathion, sumithion, phenthoate, phorate, dimethoate, and thiometon give this reaction (22); other sulfur-containing compounds such as sulfanilamide, sulfadiazine, thiobarbiturate, thiourea, carbon disulfide, and thiocarbonates do not interfere. Because an authentic sample of thiobenzophenone is not available commercially, the percentage yield of thiobenzophenone is calculated by weighing the final reaction product after purification (22) through a column and confirming the melting point and molar extinction coefficient values calculated on the basis of thiophosphates. The percentage yield of the reaction product, the applicable concentration range, and percentage determined by the described method of the declared concentrations in various formulations and technical materials are shown in Table 1. This is the first reported colorimetric method for determining organothiophosphates based on the P=S moiety. Formulations lose their activity due to conversion of P=S to P=O through auto-oxidation. The proposed method can determine the shelf-life of such formulations. The

Table 1. Results of colorimetric method for determining thiophosphates in formulations and technical materials and comparison with ISi (23) methods

Pesticide	% Yield, thiobenzophenone	Declared concn, %		Recovery of declared, %			
				Form.		Tech.	
		Form.	Tech.	ISI	P=S	ISI	P=S
A. Malathion	99.58	50.00	98.00	98.3	98.6	98.7	99.1
B. Parathion	98.45	50.00	97.00	98.5	99.0	99.5	99.2
C. Sumithion	98.95	20.00	97.00	98.3	98.0	99.2	98.6
D. Fenthion	98.25	2.00	98.00	98.7	98.5	98.1	97.9
E. Dimethoate	98.32	30.00	97.00	98.6	99.5	98.2	97.9
F. Phorate	98.48	10.00	98.00	99.7	98.7	98.9	99.7

[a] Mean results from 8 replicate determinations. Beer's law range for all pesticides listed is 35–350 μg/mL.

proposed method is most suited to those laboratories which are not equipped with sophisticated instruments for gas chromatography and liquid chromatography.

REFERENCES

(1) Getz, M. E., & Watts, R. R. (1964) *J. Assoc. Off. Agric. Chem.* **47**, 1094–1096

(2) Turner, G. R. (1974) *Analyst (London)* **99**, 431–434

(3) Ott, D. E., & Gunther, F. A. (1968) *J. Assoc. Off. Anal. Chem.* **51**, 1213–1216

(4) Morris, M. V., Vail, W. A., & Averell, P. R. (1954) *J. Agric. Food Chem.* **2**, 570–573

(5) Upham, S. D. (1960) *J. Assoc. Off. Agric. Chem.* **43**, 360–364

(6) Analytical Methods Committee Reports, "The determination of malathion residues in cereals and oilseeds" (1960) *Analyst (London)* **85**, 915–921

(7) Weisenberg, E., Gertner, S., & Scholenberg, J. (1968) *Analyst (London)* **93**, 443–444

(8) Enos, H. F., & Frear, D. E. H. (1964) *J. Agric. Food Chem.* **12**, 175–177

(9) George, D. A., Walker, K. C., Murphy, R. T., & Giang, P. A. (1966) *J. Agric. Food Chem.* **14**, 371–374

(10) Prasad, B. N., Kawale, G. B., & Joglekar, V. D. (1978) *Curr. Sci.* **47**(3), 77–79

(11) McKinley, W. P., & Read, S. I. (1962) *J. Assoc. Off. Agric. Chem.* **45**, 467–473

(12) Douglas, W. W., & Mathews, P. B. C. (1952) *J. Physiol.* **116**, 202–218

(13) Geruit, H., & Labenz, K. (1941) *Arch. Exp. Pathol. Pharmacol.* **198**, 369–389

(14) Schutz, F. (1943) *J. Physiol.* **102**, 259–268

(15) O'Brien, R. D. (1947) *Insecticides: Action and Metabolism*, Academic Press, Inc., New York, NY, p. 88

(16) Zweig, G., & Sherma, J. (1976) *Anal. Chem.* **48**(5), 66R–83R

(17) MacNeil, J. D., & Frei, R. D. (1975) *J. Chromatogr. Sci.* **13**, 279–285

(18) Hall, R. C. (1974) *J. Chromatogr. Sci.* **12**, 152–160

(19) Koen, J. G., Huber, J. F. K., Poppe, H., & den Boei, G. J. (1970) *J. Chromatogr. Sci.* **8**, 192

(20) Ryan, J. F. (1977) in *Analytical Methods for Pesticides and Plant Growth Regulators*, Vol. 9, G. Zweig & J. Sherma (Eds), Academic Press, Inc., New York, NY, p. 1

(21) Vander Velde, G., & Ryan, J. F. (1975) *J. Chromatogr. Sci.* **13**, 322–329

(22) Sane, R. T., & Kamat, S. S. (1978) *Curr. Sci.* **47**(20), 765–766

(23) A. Indian Standards Institution (1978) Emulsifiable Concentrates, IS:2567, p. 6; Dusting Powders, IS:2568, p. 7; Water Dispersible Powders, IS:2569, p. 6.
B. Indian Standards Institution (1962) Emulsifiable Concentrates, IS:2129, p. 8
C. Indian Standards Institution (1969) Technical, IS:5280, p. 15; (1979) Emulsifiable Concentrates, IS:5281, p. 8
D. Indian Standards Institution (1976) Technical, IS:7950, p. 8
E. Indian Standards Institution (1975) Emulsifiable Concentrates, IS:3903, p. 4; Technical, IS: 3902, p. 4
F. Indian Standards Institution (1976) Technical, IS:7976, p. 6

DRUGS IN FEEDS

Reverse Phase High Pressure Liquid Chromatographic Determination of Arprinocid in Animal Feeds

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A simple method is presented for determination of arprinocid in finished feeds by reverse phase high pressure liquid chromatography. The sample is extracted with 95% DMF, the major feed interferences are removed by alumina chromatography, and arprinocid is separated from the remaining interferences on the HPLC column. The peak height detected at 254 nm can be quantitated by direct comparison with the working standard.

A method for arprinocid (1), a new anticoccidial agent used in animal feeds, has recently been subjected to a successful collaborative study and subsequently adopted as an AOAC official first action method (2). In this method, arprinocid is extracted from the feed into chloroform in the presence of pH 7 buffer, transferred to 0.1N HCl, and separated from interfering substances by partitioning with hexane. The acid solution of the drug is neutralized, extracted into chloroform, injected into the normal phase column, and detected at 254 nm. The method also requires that an aliquot of the chloroform solution of the arprinocid stock standard be evaporated to dryness under nitrogen, diluted to volume in 0.1N HCl, and then handled in the same manner as the sample: neutralized, extracted into chloroform, and injected into the chromatograph. The standard is handled as an internal control for this analytical technique.

The many steps of extractions, aliquotings, centrifuging, evaporations, partitionings, and further extractions completely isolate arprinocid from most feed interferences and the high pressure liquid chromatograph was used primarily as a detection tool. Our laboratory found that arprinocid was not easily soluble in chloroform or 0.1N HCl but readily soluble in DMF, that a feed containing arprinocid could be easily extracted with 95% DMF, and that the major feed interferences could be removed by passing the filtered extract through a specified alumina column. This procedure is essentially the same as the method used in our laboratory for determining carbadox, furazolidone, nitrofurazone, and ethopabate (3).

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METHOD

Apparatus and Reagents

(a) *High pressure liquid chromatograph.*—Model 4400 equipped with U6K septumless injection system (Waters Associates, Inc., Milford, MA 01757) and Omni-Scribe recorder (Houston Instrument, Austin, TX 78753). General operating conditions: 30 μL injections, 0.05 AUFS, 254 nm detector, 10 mV recorder, 0.1 in./min chart speed, 1.5 mL/min flow rate.

(b) *Chromatographic column.*—10 cm × 2 mm id stainless steel precolumn containing μBondapak C_{18}/Corasil followed by 30 cm × 4 mm id precision bored stainless steel column containing μBondapak C_{18} (Waters Associates, Inc.).

(c) *Mobile phase.*—Acetonitrile (suitable for ultraviolet spectrophotometry or liquid chromatography) and triple glass-distilled water containing 1% acetic acid (25 + 75) and PIC Reagent B-5 (0.97 g 1-pentanesulfonic acid sodium salt/g). Mix and filter through 0.45 μm filter (Millipore Corp., Bedford, MA 01730).

(d) *Sample extraction solvent.*—Reagent grade dimethylformamide (DMF)-water (95 + 5). If DMF is colored, shake with activated carbon and filter before use.

(e) *Alumina.*—Alcoa F-20, 80–200 mesh. (Do not substitute.) Obtain from Sargent-Welch Cat. 128 (No. SC10492-005LB).

(f) *Chromatographic tubes.*—1 × 30 cm glass column constricted at one end to ca 4 mm, or 5¾ in. disposable Pasteur pipet.

(g) *Arprinocid and ethopabate stock standard solutions.*—0.40 mg/mL. Weigh 40 mg each arprinocid and ethopabate reference standards (Merck & Co., Inc., Rahway, NJ 07065) and place in separate 100 mL volumetric flasks. Dilute to volume with extracting solvent. Standards are stable if stoppered tightly.

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Figure 1. Chromatogram showing separation of arprinocid (A), 12 μg/mL; and ethopabate (E), 2 μg/mL under conditions of method.

(h) *Arprinocid working standard.*—12 μg/mL. Place 3.0 mL arprinocid stock solution into 100 mL volumetric flask and dilute to volume with extracting solvent. Prepare solution fresh daily.

(i) *Column performance test.*—Place 3.0 mL arprinocid stock solution and 10.0 mL of a 20-fold dilution of ethopabate stock solution (20 μg/mL) into 100 mL volumetric flask and dilute to volume with extracting solvent. Resulting solution contains 12 μg arprinocid/mL and 2 μg ethopabate/mL. Under conditions of method, inject onto HPLC column. Arprinocid and ethopabate peaks should be separated. See Figure 1.

Preparation of Sample Extract

Grind coarse or pelleted feeds to pass 20 mesh sieve. Weigh 10 g sample into 125 mL glass-stopper Erlenmeyer flask. Swirl while adding 5.0 mL water, and let stand 5 min. Add 50.0 mL 95% DMF, stopper tightly, shake vigorously 15 s, and let stand overnight at room temperature. Filter through rapid paper and transfer ca 15 mL onto chromatographic column containing ca 5 g alumina held in column with cotton plug. Collect eluate for HPLC injections. Quantitate drugs by comparing peak height ratios of feed extracts with working standards of about the same concentration (PH/PH'), using identical injection volumes. Use standard injection, then duplicate feed injections, followed by standard injection. Repeat if peak heights of identical injections are not the same.

Calculate percent of drug as follows:

Arprinocid, % = $[(PH/PH') \times C'(\mu g/mL) \times V(53\ mL) \times 100]/(W(g) \times 10^6)$

Results and Discussion

The work in this paper was carried out with the 6 collaborative samples submitted for the 1980 collaborative study and formulations of a diverse group of feeds spiked with approximately the same levels of arprinocid guaranteed in the collaborative samples. The value of water pretreatment and the use of 95% DMF as the extractant has been discussed in the previous paper (3).

Recoveries of arprinocid from spiked feeds indicate that DMF is a suitable extractant for the drug. The alumina chromatography used for cleanup is critical. Different types, different brands, different brands of the same type, and treated aluminas were all tried without success. Only one type and brand of alumina was successful and this was Alcoa F-20, 80–200 mesh. Substitutions cannot be made because major feed interferences will not be separated and/or recoveries of arprinocid will not be complete. Recoveries of arprinocid standards through the column in the absence of samples will be about 91%; however, in the presence of feed ingredients, 100% of the drug is eluted. Mini columns (5¾ in. disposable Pasteur pipets) work as effectively as macro columns, saving chemicals, time, and space. The eluate can then be collected in ½ dram screw-cap vials.

Alumina removes the major feed interferences, and the remaining feed interferences are separated on the HPLC column. The appearance of the peak gives some indication as to whether interferences are present. A sharp peak with good baseline resolution strongly suggests, but does not prove, that interferences are absent. It is possible for an interference to have a retention time similar to the drug in question and therefore not be visually apparent. This can be resolved by monitoring the detection of the eluting drug at 2 different wavelengths simultaneously and comparing the response ratio of the standard and sample. If the response ratio and the retention

Table 1. Recoveries of arprinocid (A) added to a corn-based feed in the presence of ethopabate (E)

Added, ppm	Sample size, g	Recovery, %
50 mL DMF Extraction		
250 ppm A 25 ppm E	10	94.0
500 ppm A 50 ppm E	10	95.4
750 ppm A 75 ppm E	10	94.0
500 ppm A 0 ppm E	10	95.0
5 mL H_2O Treatment + 50 mL DMF Extraction		
250 ppm A	—	103.6 (55 mL)
25 ppm E		99.6 (53 mL)
500 ppm A	—	103.8 (55 mL)
50 ppm E		101.0 (53 mL)
750 ppm A	—	102.5 (55 mL)
75 ppm E		98.8 (53 mL)
500 ppm A	—	103.2 (55 mL)
0 ppm E		99.2 (53 mL)
H_2O Treatment + Diln to Vol. (100 mL)		
500 ppm A 50 ppm E	20	97.0
1000 ppm A 100 ppm E	20	96.3
1500 ppm A 150 ppm E	20	96.5
1000 ppm A 0 ppm E	20	96.3

time are the same, it would strongly indicate that interferences are absent because it would be highly unlikely that different compounds would be retained and absorb at different wavelengths to the same degree. If the eluted peak was a mixture of 2 or more compounds, the ratio would be quite different from than that of the standard.

Carbadox, furazolidone, nitrofurazone, pyrantel tartrate, and ethopabate can be determined with the same sample preparation and with slight modifications of HPLC conditions but do not interfere because retention times are different. Sulfa drugs are removed by adsorption on the alumina column; amprolium, the arsenicals, tetracyclines, tylosin cannot be determined with this HPLC system. Ethopabate can interfere if certain precautions are not taken. An efficient column is necessary to separate ethopabate from arprinocid. With a mobile solvent of acetonitrile–water (containing 1% acetic acid) (25 + 75), the ethopabate elutes just ahead of arprinocid. By adding a PIC B-5 reagent to the mobile solvent, ethopabate is retained on the column longer and elutes just after arprinocid. The PIC reagent also enhances the sensitivity of the arprinocid peak by a factor of 2.5 and improves the baseline considerably.

With either mobile phase, ethopabate and arprinocid may elute together if column efficiency is low. A column performance test in the method determines if the column separates ethopabate from arprinocid.

The methodology was studied for feed ingredient interferences, recoveries of spiked samples containing arprinocid and ethopabate, linearity of response, and precision. Six unmedicated feeds carried through the procedure showed no interfering peaks at the retention time of arprinocid. A corn mash feed was spiked with 0.0025% arprinocid and 0.00025% ethopabate; 0.0050% arprinocid and 0.00050% ethopabate; 0.0075% arprinocid and 0.00075% ethopabate; and 0.0050% arprinocid only. Three sets of spiked samples were set up: One set was extracted with 95% DMF only; one set was pretreated with water before 95% DMF extraction; and the last set was pretreated with water, extracted, filtered, and diluted to a specified volume. Results are shown in Table 1.

Recoveries of arprinocid were not affected by the presence of ethopabate. Instrument response was linear from 5 to 15 ppm under conditions of the method. Recoveries of the drug not pretreated with water ranged from 94.0 to 95.4%; recoveries with water pretreatment and additive volumes ranged from 102.5 to 103.8%. These recoveries were obtained in the same range as those obtained for carbadox, furazolidone, nitrofurazone, and ethopabate for the same sample preparation. The formulation of the feed ingredients seemed to have no effect on recoveries. In all cases the sample was pretreated with 5.0 mL water and 50.0 mL 95% DMF was added for a total of 55.0 mL diluent. It was deduced that a portion of the water was adsorbed on the surface of the feed ingredients and did not act as a diluent for the drug in the extract. Calculating recoveries back to 100%, it appeared that 53 mL acted as the diluent and perhaps 2 mL water was adsorbed.

Recoveries of the drug, by the same technique including water pretreatment but with quantitative filtration to a specific volume rather than additive volumes, ranged from 96.3 to 97.0%. Lower recoveries would be expected as more manipulation is required.

If 53 mL is used in the calculation of the recoveries of arprinocid for additive volumes involving water, recoveries ranged from 98.8 to 101.0%. Extractions, potential interferences, and cleanup column effects were basically the same

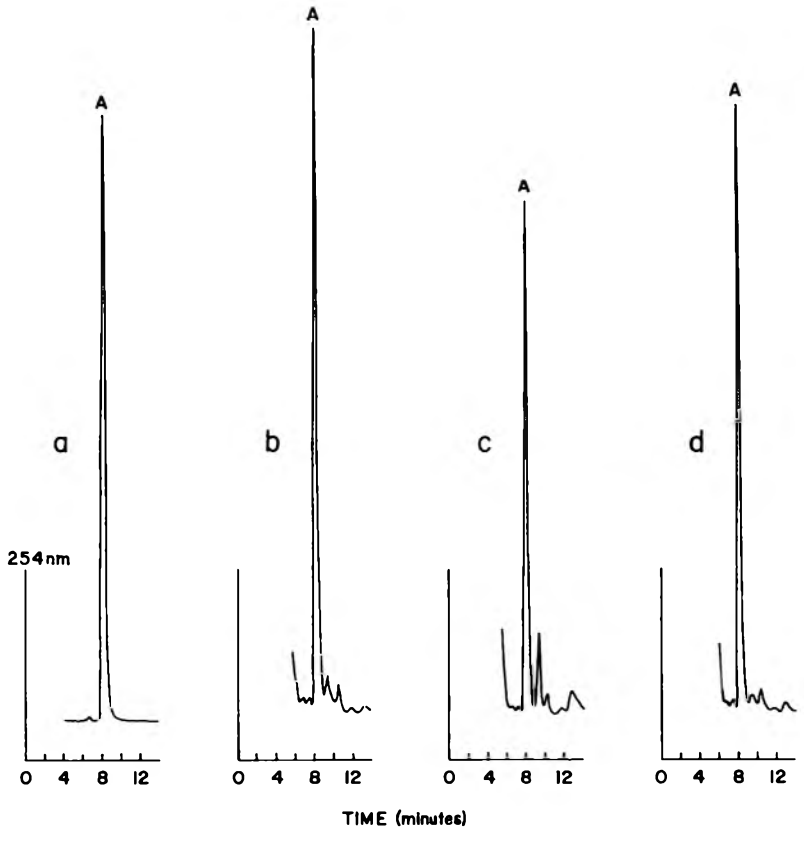

Figure 2. Chromatograms showing arprinocid (A) peaks of (a) standard, 12 μg/mL; (b) 1980 collaborative sample 2; (c) 1980 collaborative sample 4; and (d) 1980 collaborative sample 6.

in all sets, so it may be safe to assume that 2 mL water is adsorbed and 53 should replace 55 mL in the calculation. The data indicate that water pretreatment appears to be necessary for mash feeds as well as pelleted feeds to obtain good recoveries.

The 6 collaborative samples were then analyzed by the described method. Each peak was sharp with good baseline resolution. Samples and standards were monitored simultaneously at 254 and 280 nm to check for interferences. Chromatographic patterns of Samples 1, 2 and 3 were identical with the exception of arprinocid levels and a small amount of ethopabate in Sample 3. Samples 4, 5, and 6 show similar chromatographic patterns with the exception of arprinocid levels and the presence of higher amounts of ethopabate in Samples 4 and 5. Results of the collaborative samples are found in Table 2 and chromatograms of Samples 2, 4, and 6 are found in Figure 2.

Table 2. Comparison of arprinocid results for 1980 collaborative samples by normal phase HPLC collaborative method (3) and proposed reverse phase HPLC method

Formulated, %	Coll. method, %	Proposed method, %
0.0060	0.00629	0.00613
0.0070	0.00721	0.00708
0.0050	0.00526	0.00516
0.0050	0.00534	0.00513
0.0070	0.00729	0.00696
0.0060	0.00618	0.00613

In conclusion, the method presented is simple, accurate, and precise. The method does not require the extensive cleanup that is required for a normal phase column. The major feed interferences are removed by alumina chromatography and samples can be compared directly with working standards. The potential of HPLC is fully utilized not only as a detector but as a powerful tool for separation.

REFERENCES

(1) Fink, D. W., Fox, A., & Pivnichny, J. V. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1–4

(2) Changes in Methods, **42.B01-42.B05** (1981) *J. Assoc. Off. Anal. Chem.* **64,** 520

(3) Thorpe, V. A. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 981–984

Radioimmunoassay for Hygromycin B in Feeds

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A radioimmunoassay developed for the determination of hygromycin B in feed gave a coefficient of variation of 3%. Results showed 97-103% recovery of the antibiotic from various feed mixtures. Specificity studies with rabbit-anti-hygromycin B antibody showed insignificant cross reactivity with other antibiotics commonly used in combination with hygromycin B.

The present official microbiological assay for hygromycin B in feed (1) is a well established method described in detail by various authors (2-5). Because of the nature of the microbiological assay (3), sample preparation requires extraction of hygromycin B from the feed and concentration of the extract before assay. The feed is blended in a Waring blender followed by acidification, centrifugation, purification by ion-exchange chromatography, and concentration by evaporation. Samples are then assayed by a penicylinder agar diffusion method requiring 18 plates for the standard curve and 6 plates for each sample (3).

Although this assay procedure has some inherent variability, its greatest drawback is the laborious sample preparation. For this reason, a radioimmunoassay for hygromycin B in feed was developed, which offers significant advantages in time and accuracy over the existing microbiological assay procedure. Details of the procedure and experimental data are presented here.

METHOD

Apparatus

(a) *Micropipetting system.*—MLA, or equivalent, 100 μL capacity, and 100 μL capacity plastic tips.

(b) *Plastic test tubes.*—Falcon No. 2052, 12 × 75 mm.

(c) *RIA gamma tubes.*—With screw caps (Research Products International, Mt. Prospect, IL).

(d) *Gamma counter.*—1180 two-channel (Tracor Analytica, or equivalent).

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Reagents

(a) *Hygromycin B.*—Radioimmunoassay reagents were prepared by American Diagnostic, 1598 Monrovia Ave, Newport Beach, CA 92663. (1) Rabbit-anti-hygromycin B antibody (blue reagent) (AB° blue); (2) ^{125}I-hygromycin B (yellow reagent) (HB* yellow); (3) Sheep-anti-rabbit precipitating antibody (red reagent) (AB° red).

(b) *Phosphate buffered saline (PBS).*—pH 7.5, 0.01M.

(c) *Phosphate buffer.*—pH 9.0, 0.2M.

Preparation of Standard Solution

Accurately weigh suitable quantity of hygromycin B standard material to represent 100 000 units of hygromycin B activity. Place this material in 100 mL volumetric flask and dilute to volume with PBS. Refrigerate this stock solution for a maximum of 2 weeks. On day of assay, prepare working standards by volumetric dilution from the 1000 units/mL stock solution to contain 0.02, 0.04, 0.06, 0.08, and 0.10 unit of hygromycin B activity/mL PBS.

Preparation of Sample

Finished feeds.—Accurately weigh 30.0 g sample and transfer to dry blender jar. Add 750 mL 0.2M phosphate buffer, accurately measured, and blend 10 min, operating blender through powerstat setting of 7.0. Filter material from blender through Whatman No. 1 paper. Discard first 10 mL filtrate and collect ≥2 mL filtrate in clean, dry test tube. Transfer 1 mL filtrate to 10 mL volumetric flask and dilute to volume with PBS.

Hygromix®.—Accurately weigh and blend 15-20 g sample as outlined above for finished feed samples. Filter sample as above and dilute with PBS to contain between 0.05 and 0.07 unit/mL.

Assay Procedure

Into consecutively numbered plastic test tubes, set up the following (Table 1):

1. Pipet 200 μL PBS blank into each of 2 test tubes (nonspecific tubes 1 and 2).
2. Pipet 100 μL PBS blank into each of 2 test tubes for total binding (B° tubes 3 and 4).
3. Pipet 100 μL of each dilute standard solution into each of 2 tubes and 100 μL of each

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Table 1. Flow sheet for hygromycin B radioimmunoassay

Tube	Identity	Vol. buffer, std, or sample, μL	Vol. AB° blue, μL	Vol. HB* yellow, μL	Vol. AB° red, μL
TC	total count	0	0	100	0
1 & 2	nonspecific	200 PBS	0	100	100
3 & 4	B°	100 PBS	100	100	100
5 & 6	0.02 unit	100 std 1	100	100	100
7 & 8	0.04 unit	100 std 2	100	100	100
9 & 10	0.06 unit	100 std 3	100	100	100
11 & 12	0.08 unit	100 std 4	100	100	100
13 & 14	0.10 unit	100 std 5	100	100	100
15 & 16	sample 1	100 sample 1	100	100	100
17 & 18	sample 2	100 sample 2	100	100	100
19 & 20	sample 3	100 sample 3	100	100	100
21 & 22	sample 4	100 sample 4	100	100	100
etc.	etc.	etc.	etc.	etc.	etc.

sample into each of 2 tubes.

4. Pipet 100 μL rabbit-anti-hygromycin B (AB° blue) reagent into all tubes except nonspecific tubes (1 and 2). Mix each tube well with vortex mixer.

5. Pipet 100 μL ^{125}I-hygromycin B (HB* yellow) into all tubes plus an empty tube labeled TC (total count). Mix all tubes except TC tube with vortex mixer.

6. Pipet 100 μL sheep-anti-rabbit precipitating antibody (AB° red) reagent into all tubes except TC tube. Mix all tubes with vortex mixer.

Incubate at room temperature ≥20 min. Centrifuge all tubes except TC tube 10 min at ambient temperature and at ≥2000 × g. Immediately aspirate supernate, being careful not to remove any pellet. Do not aspirate TC tube. Place tubes in screw-cap gamma counting tubes and count all tubes to 10 000 counts with gamma radiation counter.

Calculations

After obtaining counts per minute (cpm) for each tube, average counts for all duplicate tubes. Calculate % bound (B):

$$\%\ B = [B\ (\text{cpm for standards or sample}) - \text{nonspecific cpm}]/[B^\circ\ \text{cpm} - \text{nonspecific cpm}] \times 100$$

Plot % B vs units/mL of standards on logit-log paper (Figure 1). Determine units/mL of sample by interpolating from standard curve.

To calculate g/ton of finished feed, proceed as follows:

g/ton = units/mL × (g/1.0 × 10^6 units) × (750 mL/30 g) × (10 mL/1 mL) × (454 g/lb) × (2000 lb/ton)

To calculate Hygromix g/lb, proceed as follows:

g/lb = units/mL × (g/1.0 × 10^6 units) × (750

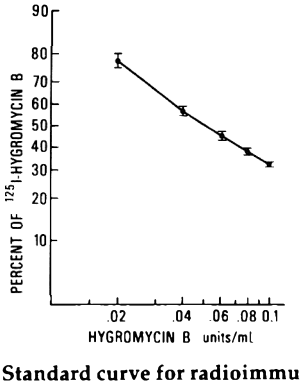

Figure 1. Standard curve for radioimmunoassay of hygromycin B. Each point represents mean ± 1 SD for 36 replicates. Line was fitted to data by hand.

Table 2. Comparison of RIA vs microbiological assays of hygromycin B internal control standard

Radioimmunoassay		Microbiological	
units/g	% Theory	units/g	% Theory
975.4	104	915.8	98
926.8	99	903.9	97
968.4	104	898.0	96
971.4	104	889.4	95
877.5	94	892.6	96
938.8	100	918.6	98
951.2	102	919.4	98
942.4	101	939.5	101
943.6	101	915.4	98
Mean[a] 943.9 ± 28.1	101 ± 3	910.3 ± 14.9	98 ± 2

[a] Mean ± 1 SD.

Table 3. Recovery of hygromycin B mixed with blank feed

Weighing	Sample	μg hygromycin B/g sample	Period 1, g/ton		Period 2, g/ton		Theor. rec., g/ton
			Day 1	Day 2	Day 1	Day 2	
1	1	15.0	13.6	13.2	13.1	13.4	13.6
1	2	15.0	13.4	13.6	13.3	13.9	13.6
2	1	15.0	13.8	13.8	13.3	13.8	13.6
2	2	15.0	13.8	13.6	13.6	13.1	13.6

mL/30 g) × dilns × (454 g/lb)

Calculate total percent binding by dividing net B° counts by the total counts. This figure is used to check the overall performance of the assay.

Results and Discussion

The standard curve depicted from a logit-log plot in Figure 1 was most linear between concentrations of 0.04 and 0.06 unit/mL; consequently, samples were diluted to read at these concentrations. Assay precision was estimated, irrespective of extraction efficiency, by measuring the potency of a hygromycin B internal control standard with a previously assigned potency of 934 units/g. For comparison, the same internal control samples were assayed by the official hygromycin B microbiological plate assay on the same day RIA was performed (Table 2). The coefficient of variation for these samples was 3.0%, with a mean value of 943.9 units/g for RIA. For the microbiological assay, the coefficient of variation was 3.0% with a mean value of 910.3 units/g. RIA overestimated the potency of the internal standard by 1.0% while the plate assay underestimated the potency by 2.5%.

When assaying feed samples that contain as little as 0.00013 g hygromycin B/g feed, obtaining a representative homogeneous sample is always a problem. To evaluate the efficiency of extraction and accuracy of RIA without the potential bias introduced by lack of sample homogeneity, a pre-weighed sample of blank feed was mixed with a known amount of hygromycin B and assayed. Samples were weighed in duplicate and duplicate aliquots were taken from each sample. Samples were assayed on 2 separate days at 2 different time periods. Three months separated assay period 1 and assay period 2. Recoveries of the 16 samples prepared to contain 13.6 g hygromycin B activity/ton feed are shown in Table 3. The mean percent recovery for period 1 was 100 ± 1% standard deviation (SD). The mean percent recovery for assay period 2 was 99 ± 2% SD. Overall mean percent recovery for both assay periods was 99 ± 2% SD.

Recovery experiments were also performed under conditions that did not control the variable of sample homogeneity. One lot of laboratory-prepared hygromycin B pre-mix (2.4 g/lb) and 2 lots of laboratory-prepared hygromycin B finished feeds (12 g/ton) were extracted and assayed by RIA. Results of these recovery studies are shown in Table 4. The mean recovery was 97% ± 9% SD for the Hygromix pre-mix, 98% ± 5% SD for Hygromix in SW-30 (an experimental swine ration prepared at the Lilly Agricultural Station) feed, and 98% ± 8 (SD) for Hygromix in CK-17 (an experimental chick ration prepared at the Lilly Agricultural Station) feed. No activity was detected in the CK-17 and SW-30 blank feeds containing no hygromycin B.

In a similar recovery study with a commercially prepared feed pre-mix blended to contain 8.0 g hygromycin B activity/lb, samples were assayed by both radioimmunoassay and the microbiological plate assay. Mean recovery for RIA (6 determinations) was 103% ± 1% SD; mean recovery for plate assay (6 determinations) was 108% ± 3% SD.

Cross-reactivity of the hygromycin B assay with other antibiotics commonly found in feed (monensin, tylosin, bacitracin, streptomycin, and chlortetracycline) was measured by determining the amount of antibiotic required to produce 50% inhibition of ^{125}I-hygromycin B binding to antibody. The percentage of cross-reactivity was determined as relative efficiency of competition

Table 4. Recovery [a] of hygromycin B from laboratory-prepared lots of hygromycin B pre-mix and finished feeds

Sample	Amt added	Mean % rec.	SD
Hygromix pre-mix	2.4 g/lb	97	9
Hygromix in SW-30	12.0 g/ton	98	5
Hygromix in CK-17	12.0 g/ton	98	8
CK-17 without hygromycin B	0	0	0
SW-30 without hygromycin B	0	0	0

[a] Each recovery is the mean for 5 samples.

Table 5. Recovery of hygromycin B in the presence of other antibiotics

Antibiotic added	Hygromycin B added, μg/mL	Hygromycin B recd, μg/mL	Rec., %
Monensin, 1000 g/ton	0.080	0.080	100
Monensin, 1000 g/ton	0.020	0.020	100
Tylosin, 1000 g/ton	0.080	0.081	101
Tylosin, 1000 g/ton	0.020	0.020	100
Bacitracin, 95 units/mL	0.060	0.060	100
Bacitracin, 95 units/mL	0.040	0.038	95
Streptomycin, 91 units/mL	0.060	0.061	102
Streptomycin, 91 units/mL	0.040	0.039	98
Chlortetracycline, 93 units/mL	0.060	0.062	103
Chlortetracycline, 93 units/mL	0.040	0.038	95

by the other antibiotics compared with hygromycin B. Monensin, tylosin, bacitracin, streptomycin, and chlortetracycline all showed less than 0.1% cross-reactivity with the hygromycin B antibody. In addition to the percentage of cross-reactivity study, specificity was also verified by a combination antibiotic recovery study. Concentrations of hygromycin B varying from 0.02 to 0.08 mg/mL were combined with monensin (1000 g/ton), tylosin (1000 g/ton), bacitracin (95 units/mL), streptomycin (90 units/mL), or chlortetracycline (95 units/mL), and the percent recovery of hygromycin B was determined (Table 5). Recoveries ranged from 95 to 103% of theory. No cross-reactivity was detected with any of the antibiotics commonly used in conjunction with hygromycin B in either the combination antibiotic recovery study or the competitive binding study.

Conclusion

The radioimmunoassay described herein was more rapid, more sensitive, and less laborious than the microbiological plate assay (1) for hygromycin B in feed. The precision of the assay was established by use of an internal control standard; the coefficient of variation for 9 determinations was 3%. Recovery experiments gave results of 99, 97, 98, 101, and 103% for the recovery of hygromycin B added to a preweighed blank feed, laboratory-prepared premix, laboratory-prepared finished feeds, and a commercially prepared finished feed, respectively. Specificity studies showed that the rabbit-anti-hygromycin B antibody had a cross-reactivity of <0.1% with other antibiotics commonly used in combination with hygromycin B. Thus, RIA for hygromycin B in feeds overcomes many of the difficulties commonly encountered with the microbiological plate assay, i.e., laboriousness, inherent variability, and low recoveries. Because of increased sensitivity, RIA eliminates the need for column purification and concentration steps required by the microbiological assay. Disadvantages of the hygromycin B assay include the necessity for specialized equipment and the relatively short life (6 weeks) of the ^{125}I-labeled hygromycin B reagent.

References

(1) *Official Methods of Analysis* (1970) 11th Ed., AOAC, Arlington, VA, secs. **38.183-38.187**
(2) Dennin, L. J. (1963) in *Analytical Microbiology*, F. Kavanagh (Ed.), Academic Press, New York, NY, pp. 303-307
(3) Hilty, W. W. (1960) *J. Assoc. Off. Agric. Chem.* **43**, 213-223
(4) Wright, W. W. (1965) *J. Assoc. Off. Agric. Chem.* **48**, 109-110
(5) Hilty, W. W. (1965) *J. Assoc. Off. Agric. Chem.* **48**, 264-267

Direct Spectrophotometric Determination of Arprinocid in Premixes: Collaborative Study

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A spectrophotometric analytical method for the determination of arprinocid [9-(2-chloro-6-fluorophenylmethyl)-9*H*-purin-6-amine] in premixes has been evaluated in an interlaboratory collaborative study. The samples were prepared in pairs over the concentration range 10.1-16.2% arprinocid with 2 similar materials as carriers for the drug, and were distributed to 16 laboratories. Duplicate analyses were obtained within the overall paired sample design by a protocol which obviates data censoring and allows for statistical treatment according to both Youden and Steiner guidelines. Each collaborator was requested to run 1 determination on each of 16 differently numbered samples in groups of 8 on 2 different days. In this analytical procedure, the drug is extracted into $CHCl_3$ and transferred into 0.1N HCl for measurement at 258 nm after isolation by liquid-liquid partitioning. The means of the analyses reported by the collaborators ranged from 96.9 to 101% of the true concentration of arprinocid and were not statistically significantly different ($P > 0.1$) from the true values. The coefficient of variation at the 12.2% formulation concentration level was 3.6%. The collaborative data were normally distributed ($P < 0.01$) with a mean, median, and mode of 99.2, 99.2, and 100.0% recovery, respectively, and a standard deviation of 3.72%. In this distribution, 80% of the results were within the range 95.1-104.1% recovery. The systematic standard deviations of the 4 unit blocks (s_b) were each <0.4% arprinocid and the analysis of variance demonstrated that laboratory bias is not statistically significant ($P > 0.05$). There is no statistically significant difference in either accuracy ($P > 0.5$) or precision ($P > 0.05$) between days, and the ruggedness test confirms that none of the 7 procedural variations which were selected for study has a significant effect on the analysis. This method has been adopted official first action.

The coccidiostat arprinocid, 9-(2-chloro-6-fluorophenylmethyl)-9*H*-purin-6-amine, can be determined in medicated feed in the concentration range 0.0045-0.0080% by either a colorimetric (1) or a high pressure liquid chromatographic (HPLC) (2, 3) analytical procedure. This drug is formulated as a premix which contains 12% arprinocid for blending into the feed in the proportions of 1 lb premix/ton feed to furnish a final finished feed concentration of 0.0060% arprinocid.

Although the premix can be analyzed by both the colorimetric and HPLC methods, this sample does not require the extensive preparatory separations involved in these methods for feeds because of the high drug concentration and the nature of the extraneous interferences. A direct spectrophotometric measurement for this sample would also be more rapid because it would entail neither the reaction time for color development of the former method nor the chromatographic retention time associated with the latter. Arprinocid premixes have also been analyzed by an equally rapid differential pulse polarographic technique (4). In consideration of the most practical and rugged analytical method that can be universally applied in the greatest number of laboratories, the present direct spectrophotometric analytical method was selected for collaborative testing. The statistical treatment of this study is based on single determinations of paired samples as advocated by Youden (5), but with the additional feature of including duplicates as recommended by Steiner (5) in an experimental design which obviates the possibility of data censoring which is of concern to Youden whenever replicates are requested.

Arprinocid in Premixes Spectrophotometric Method Official First Action

Principle

Arprinocid is extd from premix into $CHCl_3$ and transferred into 0.1N HCl. Interferences are removed by partitioning with CCl_4 and arprinocid is detd by direct spectrophotometric measurement at 258 nm.

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Table 1. Ruggedness test: factors studied and assigned altered values

No.	Factor	Nominal	Change
1	extraction time	(A) 20 min	(a) 15 min
2	filtration volume	(B) 20 mL	(b) 15 mL
3	evaporation temperature	(C) 50°C	(c) 70°C
4	evaporation atmosphere	(D) N_2	(d) air
5	solvent acidity	(E) 0.10N HCl	(e) 0.09N HCl
6	dissolution time	(F) 20 min	(f) 15 min
7	analytical wavelength	(G) 258 nm	(g) 260 nm

Reagents and Apparatus

(a) *Spectrophotometer.*—Suitable for measuring *A* at 258 nm.

(b) *Hydrochloric acid.*—0.1N. Dilute 8.3 mL HCl to 1 L with H_2O.

(c) *Arprinocid standard solution.*—Accurately weigh 0.12 ± 0.01 g arprinocid std (Merck Sharp & Dohme, Rahway, NJ 07065) and dissolve in 100.0 mL $CHCl_3$.

Extraction

Accurately weigh 1.00 ± 0.05 g premix and transfer to 125 mL g-s erlenmeyer. Add by pipet 100.0 mL $CHCl_3$ and mech. shake 20 min. Filter ca 20 mL ext thru Whatman No. 42 paper, covering funnel to prevent evapn of solv. Transfer by pipet 3.00 mL filtrate and 3.00 mL arprinocid std soln to sep. 50 mL centrif. tubes. Place tubes in ca 50° H_2O bath and evap. solv. to dryness under N stream.

Partitioning

Add by pipet 5.0 mL CCl_4 followed by 25.0 mL 0.1N HCl to the tube and mix 20 min in ultrasonic bath. If residue is not dissolved, addnl shaking is required. Centrifuge 5 min at 2000–2500 rpm.

Determination

Transfer by pipet 5.00 mL upper aq. phase to 100 mL vol. flask, dil. to vol. with 0.1N HCl, and mix. Measure absorbance of the sample (A) and std (A') at 258 nm vs 0.1N HCl in ref. cell.

$$\text{Arprinocid, \%} = (A/A') \times (W'/W) \times 100$$

where W = g sample; and W' = g std.

Collaborative Study

Preparation of Collaborative Samples.—The 4 statistical unit blocks were prepared at concentrations of 10.1, 12.2, 14.2, and 16.2% arprinocid to represent a range which includes the expected commercial premix concentration. The members of each pair were prepared with 2 closely similar materials as carriers for the drug: Wheat midds served as the carrier for 1 member of each pair and rice hulls as its complement. The arprinocid in each collaborative sample was blended onto its appropriate carrier with 2.5–3.5% soybean oil as a binder and 0.5% ethoxyquin antioxidant stabilizer to inhibit oxidative polymerization of the oil. The batch sizes of the 8 preparations ranged from 250 to 800 g; they were blended 15–20 min in a bench-top Hobart mixer by procedures typical of standard accepted practice.

Ruggedness Test.—In advance of distributing the collaborative samples, this method was evaluated by the conventional ruggedness test to examine the effects of reasonable, minor variations in the procedure. The 7 factors selected for study and their altered values are listed in Table 1. No control condition was assigned. A premix containing 12.2% arprinocid was analyzed by the standard plan of 8 combinations which introduces several of the changes simultaneously. The results of this ruggedness test confirmed that none of the variations listed in Table 1 has a significant effect on the analysis.

Laboratory Participation.—One month before the collaborative samples were circulated, each potential collaborator received 2 practice samples in an attempt to identify outlying laboratories and to gain experience with the method in the design recommended by Steiner: One of the practice samples was labeled to contain 12% arprinocid and the other was sent as an unlabeled unknown. Its concentration, 14%, was provided in a sealed envelope attached to the sample, to be opened after completion of the analysis. The Associate Referee recommended that an accuracy

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The recommendation of the Associate Referee was approved by the General Referee and Committee G and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 65, March issue (1982) for detailed reports.

of ≤5% relative error be established as a criterion for laboratory participation. No comments were received. The final sample of 16 laboratories included representatives of 3 foreign countries (i.e., Australia, England, and Holland) in addition to 13 domestic participants.

Experimental Design.—Each of the 8 collaborative samples was sent in duplicate bearing different sample numbers. Hence, because the collaborators were unaware of receiving duplicates, censoring was eliminated in this collaborative design. The 16 samples were divided into 2 groups of 8 to be analyzed on 2 days such that the duplicates were treated on different days. Although it is recognized that this experimental design confounds between-day effects with sample duplicates, it nevertheless is designed to reveal the possibility of improved results with added experience. In addition, because each member of the 4 statistical pairs was analyzed on each day, this design does not sacrifice any statistical information furnished by the overall paired statistical design.

Distribution of Samples.—The 8 samples for each day were numbered in random order with the aid of a computer-generated table of random numbers (6). Each collaborator was supplied with 3.5 ± 0.1 g of each premix sample, 0.5 g analytical reference standard arprinocid, a copy of the absorption spectrum in 0.1N HCl, and the structure of the drug for information. The collaborators were told only that all samples contained the drug in the concentration range 9–18% arprinocid. Each collaborator was requested to perform a single analysis on each of the 16 premixes in 2 groups of 8 samples on each of 2 days. The collaborators were urged to prepare duplicate standards on each day to confirm that these do not differ by more than ±0.005 absorbance unit and to repeat the measurement of an analytical standard at the end of the 8 analyses to check for any possible instrument drift.

Results and Discussion

The 2 well known principles of collaborative statistical testing have been clearly compared by Horwitz in his Foreword to Youden and Steiner's *Statistical Manual of the AOAC* (5): The difference in the techniques of these 2 statisticians concerns the use of duplicates. Youden's design calls for single determinations on paired unit blocks to separate systematic from random error. He emphasizes that duplication increases the number of degrees of freedom for the precision error, but adds nothing to the systematic error estimates. Youden stresses the value of only single determinations because he is concerned about the possible censoring of results that could occur when a chemist knows that he is repeating his analysis. Conversely, Steiner's guidelines call for the use of duplicates as a factor in the analysis of variance computations and to establish the homogeneity of the residual variance. His manual does not consider the possibility of censoring. The present study incorporates the attributes of both experimental designs in a collaborative study in which duplicates are obtained within the overall framework of a paired sample design. The possibility of censoring is eliminated by distributing the duplicates under different sample numbers and requesting single determinations on each sample. Because the collaborators are not aware of receiving duplicates, censoring is not a factor. This experimental design allows for data treatment according to both Youden and Steiner guidelines. The 256 (2^8) analytical results of the collaborative study are presented in Table 2, which shows that the means of the recoveries of arprinocid on each sample ranged from 96.9 to 101%.

The 2-sample charts for the 4 unit blocks are presented in Figure 1. That the averages of the duplicates are used for each point of each sample of the pairs gives more confidence to these points than is usually available from conventional charts which are based on single measurements. The points are predominantly found in quadrants I and III, representing +,+ and −,− results:

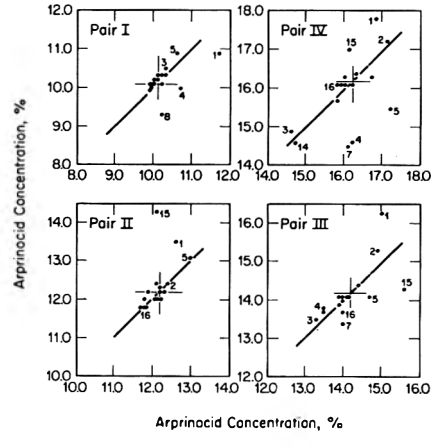

Figure 1. Two-sample charts for the 4 statistical unit blocks. Each pair consists of the same concentration of arprinocid blended onto 2 different materials as carriers. Numbers beside the points identify the collaborators; only certain laboratories are identified. The reference values are indicated by the cross-hatch near the center of each chart.

Table 2. Collaborative results for the determination of arprinocid in premixes (% arprinocid found)

	Pair I				Pair II				Pair III				Pair IV			
	Wheat midds		Rice hulls		Wheat midds		Rice hulls		Wheat midds		Rice hulls		Wheat midds		Rice hulls	
Coll.	Spl 4 Day I	Spl 13 Day II	Spl 6 Day I	Spl 15 Day II	Spl 1 Day I	Spl 11 Day II	Spl 7 Day I	Spl 12 Day II	Spl 3 Day I	Spl 9 Day II	Spl 8 Day I	Spl 16 Day II	Spl 2 Day I	Spl 10 Day II	Spl 5 Day I	Spl 14 Day II
1[a]	10.3	13.0	10.1	11.6	12.5	12.6	12.9	14.1	14.1	15.9	15.1	17.5	15.6	18.0	17.7	17.8
2	9.95	10.1	10.0	10.1	12.1	12.6	12.1	12.6	14.8	15.0	14.8	*15.7*	17.2	16.9	16.5	*17.8*
3	9.95	10.7	10.3	10.7	11.6	11.9	11.5	12.1	13.1	13.5	13.3	13.7	14.4	14.7	14.3	15.4
4	10.8	10.6	9.82	10.2	12.8	11.0	12.0	12.3	13.8	13.1	12.9	14.6	16.9	15.4	13.8	15.4
5	10.9	10.3	*11.2*	10.6	12.9	13.0	13.3	12.9	14.6	14.8	14.0	14.1	16.9	17.5	15.1	15.8
6	10.1	9.68	10.3	9.83	12.0	11.5	12.3	11.6	14.1	13.7	14.0	13.8	16.0	15.6	15.8	15.5
7	10.2	9.97	10.3	9.99	12.3	11.9	12.4	11.9	14.0	14.0	14.1	12.7	16.2	16.0	13.8	15.2
8	10.1	10.2	10.1	*8.53*	11.9	12.5	13.2	*10.7*	13.3	14.6	14.0	13.9	16.1	15.8	16.3	16.3
9	10.3	10.3	10.2	10.3	12.1	12.0	12.3	12.3	14.2	14.6	14.2	14.6	16.1	16.5	16.4	16.4
10	10.2	10.1	9.96	10.3	12.4	12.1	12.2	12.2	14.3	13.9	14.1	14.1	16.2	15.7	16.0	16.2
11	10.0	9.88	10.1	10.0	12.1	11.9	12.2	12.1	13.9	14.1	13.9	14.3	15.8	16.0	16.0	16.1
12	10.5	9.87	10.4	10.2	12.1	12.0	12.5	12.2	14.0	14.1	13.9	14.2	16.5	15.8	16.1	16.4
13	10.0	10.0	10.1	10.3	12.2	12.0	12.2	11.8	14.1	13.7	14.0	14.2	16.4	15.9	16.1	16.0
14	9.92	9.78	10.0	9.90	11.5	11.8	11.7	11.8	13.3	13.7	13.4	13.9	*14.3*	15.0	14.1	15.0
15	10.1	10.0	10.4	10.2	11.9	12.2	*15.9*	12.7	14.5	*16.6*	14.4	14.2	16.1	16.1	16.2	17.7
16	10.0	9.84	10.0	10.0	11.7	11.8	11.8	11.8	13.9	14.0	13.9	13.5	15.8	15.8	16.1	16.0
Av., %[a]	10.1		10.2		12.1		12.2		14.0		14.0		16.0		15.7	
CV, %	2.9		2.1		3.5		3.6		3.6		3.3		4.2		5.6	
Formulated concn, %	10.1		10.1		12.2		12.2		14.2		14.2		16.2		16.2	
Arprinocid rec., %	100		101		99.2		100		98.6		98.6		98.8		96.9	

[a] Outlying laboratory 1 and individual italic results excluded.

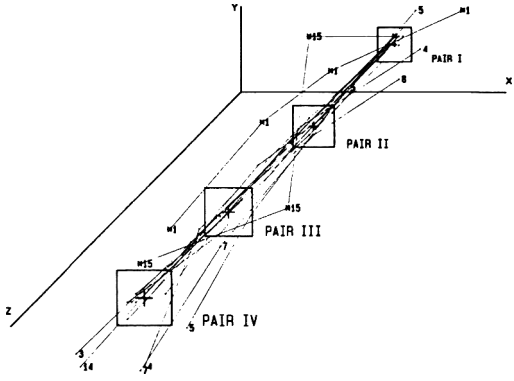

Figure 2. Composite of the 2-sample charts. Abscissas and ordinates of the pairs remain the same as Figure 1. Frames around the reference concentrations in each plane show the bounds of ±1 standard deviation of the reference values of each sample.

of the 64 total points, 82% of those not located on the borders between quadrants are found in quadrants I and III. The usual elliptical pattern around a major axis at 45° is clearly evident and the points are also generally clustered near the reference concentrations. Only certain laboratories removed from the clusters are identified in this figure. The 4 points for Collaborator 1 lie well out of the clusters in quadrant I and distant from the 45° lines. This pattern is clear evidence of both a systematic error which is large in comparison with the other collaborators and which is working in combination with an atypically large precision error. It is interesting that the scatter of data points from the 45° lines in these 2-sample charts increases with arprinocid concentration, a pattern which is also reflected in the coefficients of variation included in Table 2.

Figure 2 is a composite of the 2-sample charts which serves to highlight those points which are consistent among the 4 charts. The pattern of Collaborator 1 is clearly characterized by its linearity and its location well removed from the cluster of lines. The large precision error of Collaborator 15 is also evident in the pattern of its discontinuous line.

Rejection of Outliers.—The results of ranking the collaborative analyses are presented in Table 3. The report of Collaborator 1, which ranked No. 14, 15, or 16 on 11 of the samples, also included the highest analytical result on half of the 16 premixes. The total rank for this laboratory, 217, is well beyond the approximate 5% 2-tail limits for ranking 16 collaborators with 16 samples, which are 190 and 81. With this ranking, which is also apparent in the 2-sample charts (Figures 1 and 2), Laboratory 1 was not included in the further analysis of the data because of this abnormally large systematic error.

Although Laboratories 5 and 14 also received total rankings beyond the statistical limits, the results from these collaborators were retained on the basis of their average recoveries over the 16 samples of 104 and 95%, respectively. The total ranks for Laboratories 3 and 16 are just beyond the criteria for rejecting an outlying laboratory. However, the ranks for these collaborators generally reporting low results are within 1.5 units of the statistical limit. In addition, the points for these collaborators, as well as for Collaborator 5, are not consistent among the four 2-sample charts. Hence, because they are not clearly identified unambiguous outliers, Collaborators

Table 3. Ranked collaborator results

Coll.	Sample 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total rank
1	14	3	10	12.5	16	7.5	13	16	15	16	14.5	16	16	15.5	16	16	217
2	8.5	16	16	2.5	15	4	5	15	14	14	14.5	13	9.5	15.5	7	15	184.5
3	2	2	1	2.5	4	12	1	2	2	1	6	7.5	15	3.5	15	3	79.5
4	15	14.5	4	15	1.5	1	4	1	1	3	1	11.5	14	3.5	9	13.5	112.5
5	16	14.5	15	16	5	16	15	8.5	13	15	16	15	12.5	6	14	7.5	205
6	6	6	10	8	6	12	9.5	8.5	4	4	2	2	1	5	2	4	90
7	12	10.5	7.5	10.5	1.5	12	11	11.5	7.5	10.5	6	6	6	2	4	1	119.5
8	4.5	8	2.5	8	13	7.5	14	8.5	11.5	7	13	1	11	11	1	5.5	127
9	8.5	8	12	12.5	14	10	9.5	13	11.5	13	9	11.5	12.5	12.5	12	13.5	183
10	13	10.5	13	10.5	7.5	2	7	11.5	6	5	11	9.5	9.5	10	12	7.5	145.5
11	8.5	4.5	5.5	5	7.5	7.5	7	5	9.5	10.5	6	7.5	5	9	5.5	12	115.5
12	8.5	13	7.5	14	10	14.5	12	5	9.5	7	9	9.5	4	12.5	9	10	155
13	11	12	10	5	10	7.5	7	8.5	4	9	9	4	7.5	7.5	12	5.5	134
14	1	1	2.5	1	3	4	2	3	4	2	3.5	4	2	1	3	10	42.5
15	4.5	8	14	8	12	14.5	16	14	16	12	12	14	7.5	14	9	10	185.5
16	3	4.5	5.5	5	10	4	3	5	7.5	7	3.5	4	3	7.5	5.5	2	80

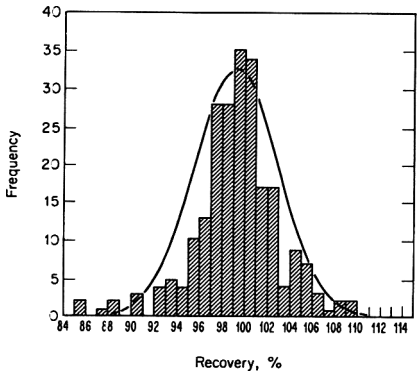

Figure 3. Frequency distribution of data. All results normalized to percent recovery. Class width: 1% recovery. Normal error curve shown is of mean 99.2% recovery and standard deviation 3.72% recovery.

3 and 16 were also retained in the following statistical analysis.

After excluding outlying Laboratory 1, there are 8 individual laboratory results remaining which are beyond the limits established by the normal law of errors as evaluated by Dixon's test. These are indicated in Table 2; each of the calculated values of r_{22} for these data exceeded the critical value of r_{22} for 15 measurements, which is 0.53. Because each of these ratios is beyond the criterion for rejection with a 1 in 20 probability of a wrong decision, these 8 individual outlying results were excluded. It is not surprising to find 8 statistical outliers in a sample of 240 data points, and these 8 are uniformly distributed across the concentration range, with 2 individual outliers found in each of the 4 unit cells. It is significant that 3 of the collaborators (2, 8, and 15) each accounted for 2 of the 8 outliers. Elimination of the 2 outliers reported by Collaborator 15 (on pairs II and III) also serves to straighten this collaborator's line in Figure 2.

Homogeneity of Variation Between Replicates.—The homogeneity of the residual variance can be established in this collaborative study because each of the members of the 4 unit cells was run in duplicate by all collaborators. This calculation is based on the variance of the range of duplicate values for each laboratory and sample after omitting the outliers. The maximum variance found, s^2_{max}, was 1.62 and the sum of the 112 variances, Σs_k^2, was 15.95, yielding a ratio $s^2_{max}/\Sigma s_k^2 = 0.10$. This ratio is below the critical limit of Cochran's test for homogeneity with a 1 in 20 probability of a wrong decision, confirming that the data may be considered homogeneous.

Statistical Distribution of the Data.—Figure 3 presents a frequency distribution for all of the results exclusive of the 8 individual outliers and of Collaborator 1 (232 data points) normalized to percent recovery on each sample and using a class width of 1% recovery. The mean, median, and mode of this distribution are 99.2, 99.2, and 100.0% recovery, respectively, and a small negative skew is apparent in the figure (i.e., 7 analyses ≤89% recovery). In this distribution, 80% of the values lie within the range 95.1–104.1% recovery. The standard deviation of this distribution is 3.72% recovery, yielding a 3.75% coefficient of variation.

Figure 3 also includes a normal distribution of a mean of 99.2% and a 3.72% standard deviation for comparison with the actual data. These data were tested for normalcy according to Laitinen (7). Figure 4 shows the plot on normal probability paper of the cumulative percent frequency as a function of percent recovery using a class width of 2% recovery. The linearity of these data for recoveries ≥92% demonstrates that the distribution is normal, and the positive deviation at concentrations below this range results from the small negative skew. The χ^2 test was also used to determine if this distribution follows the normal curve. The calculated χ^2-value for these data over the 11 classes of Figure 4 which cover recoveries ≥91% compared to the expected

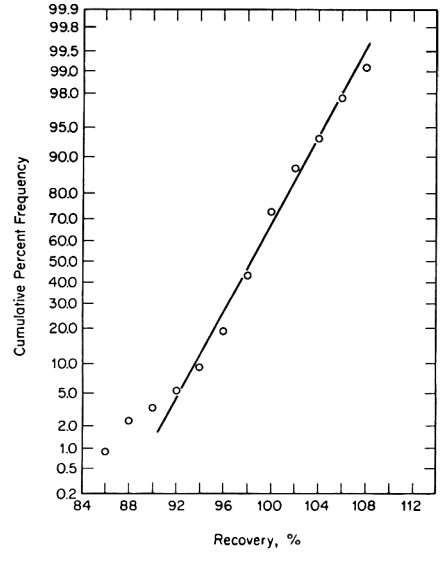

Figure 4. Normal probability plot of data. Cumulative percent frequency as a function of percent recovery. All results normalized to percent recovery. Class width: 2% recovery.

Table 4. Results of statistical analysis of collaborative results for arprinocid

Pair	Amt. present, %	Av. found, %	Diff., %	95% Conf. limits	Precision ($\sqrt{2}S_r$)	Systematic (S_b)	Distribution (S_d)	F-value ($S_d^2/2S_r^2$)	DF	CV, %	Rec., %	$(\bar{T} - R_T)/2$
I	10.1	10.2	0.1	10.1–10.3	0.24	0.10	0.22	0.84	12	2.5	101	0.050
II	12.2	12.1	0.1	11.8–12.4	0.16	0.33	0.48	9.0	12	3.6	99.2	−0.050
III	14.2	14.0	0.2	13.7–14.3	0.31	0.36	0.56	3.3	12	3.4	98.6	−0.150
IV	16.2	15.9	0.3	15.5–16.3	0.88	0.29	0.74	0.71	12	5.0	98.2	−0.250

frequencies of the normal distribution is 18.75. This is less than the critical χ^2 at the 1% level of significance for 10 degrees of freedom, confirming that the assumption that this distribution is indeed random and cannot be rejected ($P < 0.01$).

Statistical Results.—Table 4 summarizes, in standard form, the results of the statistical study based on the averages of each pair of the blind duplicates. The precision standard deviation, $\sqrt{2}s_r$, was computed from the differences of the paired samples, the distribution standard deviation, s_d, from the totals of the pairs, and the standard deviation of the distribution of systematic errors, s_b, from the relationship $s_d^2 = 2s_b^2 + s_r^2$. Using the 26 differences between duplicates available from each pair, the independent estimates of s_r are 0.30, 0.49, 0.59, and 0.69% arprinocid for pairs I–IV, respectively. Only the last of these values is less than the corresponding $\sqrt{2}s_r$ value in Table 4. That the other 3 values are greater than the corresponding $\sqrt{2}s_r$ values correlates with the fact, as explained by Youden, that censoring could not have occurred because the collaborators were unaware of receiving duplicates in this study. For 12 degrees of freedom, the calculated F-value variance ratios for pairs II and III exceed the critical F at the 5% level of significance, 2.69. However, the calculated F-values for pairs I and IV are less than the critical F even at the 10% level ($P > 0.1$). These ratios also substantiate the absence of systematic error in the method because s_d^2 is $< 2s_r^2$ half the time. In addition, the 2 statistically significant s_b values are each <0.4% arprinocid and are <3% of the true concentrations in these pairs. The coefficients of variation listed in Table 4 were computed in a standard manner from all of the 58 individual analyses at each concentration level. The values show a trend of increasing coefficient with drug concentration which is also illustrated by the plot of s^2 as a function of arprinocid concentration shown in Figure 5. In this figure, the smooth curves which are drawn through the data show that s_r^2 increases rapidly as the amount present gets larger, and that (with the exception of the most concentrated samples) the s_b^2 curve generally lies above the s_r^2 curve. The shape of the s_r^2 curve is in accord with the qualitative appearance of the 2-sample charts in Figures 1 and 2.

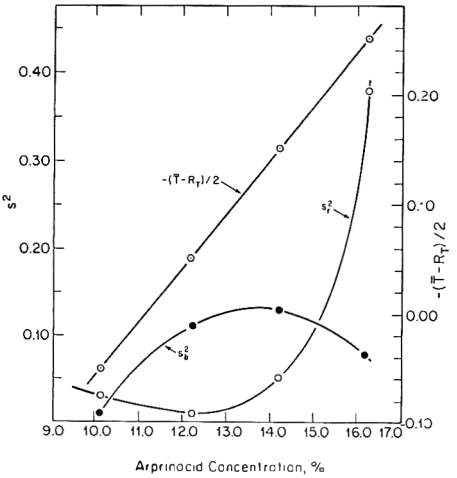

Figure 5. Accuracy and precision variances and bias of systematic error, $(\bar{T} - R_T)/2$, as a function of arprinocid concentration. Left ordinate: ●, S_b^2, ○, s_r^2. Right ordinate: ⊙, $-(\bar{T} - R_T)/2$.

Accuracy.—Because the true reference concentration of arprinocid is known in this collaborative study for each unit cell, Student's t-test can be used to test for systematic error in this method. For pairs I, II, III, and IV the calculated values of t are 1.2, 0.53, 1.35, and 1.72 for corresponding s_T values of 0.31, 0.68, 0.80, and 1.05%, respectively. The t-values are less than the critical t at the 10% level of significance for 12 degrees of freedom, demonstrating that there is no statistically significant difference ($P > 0.1$) between the average analytical results and the true (reference) values. Figure 5 also includes a plot of the values of $(\bar{T} - R_T)/2$ for each pair (which are listed in Table 4) to show the effect of the amount of arprinocid present in the sample on the systematic error of the method: The fig-

Table 5. Analysis of variance of collaborative results for arprinocid

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between labs	95.6	14	6.83 (MS_L)	1.13 (MS_L/MS_{LS})
Between samples	1100	7	157	26.0
Laboratory–sample interaction	592	98	6.04 (MS_{LS})	0.33 (MS_{LS}/MS_O)
Between replicates	1900	105	18.1 (MS_O)	

ure reveals the perfectly linear increase of systematic error with sample concentration (correlation coefficient >0.99).

Analysis of Variance.—Table 5 presents the analysis of variance table of the collaborative data. These statistics ignore the paired unit cells of Youden but include the laboratory-sample interaction term which is provided by the duplicates in this experimental design as recommended by Steiner. However, the statistical computations presented in this table must be considered an approximation because an equal number of observations are not provided for the 8 of the 120 laboratory-sample combinations which contain the individual outliers. Nevertheless, the *F*-ratios between laboratories and for laboratory-sample interaction are both less than the critical *F*-values for the corresponding degrees of freedom at the 5% level of significance. These results, then, confirm that neither a statistically significant ($P > 0.05$) laboratory bias nor interaction exists.

Difference Between Days.—Table 5 cannot include a between-days variance ratio because this effect is confounded with the variance between duplicates in this experimental design. Accordingly, the difference in accuracy and precision between days was evaluated by 2 independent statistical tests. The mean recovery over all the individual analyses on day I ($n = 117$) and on day II ($n = 115$) was 99.2 and 99.1%, respectively, and the corresponding standard deviations were 4.03 and 3.53%, yielding coefficients of variation of 4.06 and 3.56% for the 2 days. A comparison of the means of these 2 distributions using the appropriate form of the *t*-test in this sensitive experiment which provides 230 degrees of freedom yields a *t*-value of 0.2. This value is less than the critical *t* even at the 50% level of significance for an infinite number of degrees of freedom, 0.674. Hence, there is no statistically significant difference ($P > 0.5$) between the means of the analyses on the 2 days. The differences in the standard deviations between days were compared via the conventional *F*-test variance ratio. The calculated *F*-ratio, 1.14, is less than the critical *F* at the 5% level of significance, demonstrating that precision did not change ($P > 0.05$) between the days. Although an improvement in precision might have been expected on the second day because the collaborators would have more experience with the method, it is probable that their prior experience 1 month earlier with the 2 practice samples served to offset this effect.

Spectral Properties and Characterization of the Method.—In the present procedure for the determination of arprinocid in premixes, the drug is extracted from the sample into $CHCl_3$ and transferred into 0.1*N* HCl for measurement. The solubility of this compound is 3.1 mg/mL in the former solvent and 2.6 mg/mL in the latter. Further partitioning of the final analytical solution with CCl_4 ensures that interferences are separated to provide a sufficiently pure sample for direct spectrophotometric measurement. The ultraviolet absorption spectrum used for analytical measurement is presented in Figure 6, showing the λ_{max} at 258 nm ($\epsilon = 1.59 \times 10^4$ L/mole-cm) which serves as the analytical wavelength. These spectral characteristics reflect electronic transitions localized primarily on the adenine chromophore of this compound, essentially unperturbed by the 2-chloro-6-fluorobenzyl-substituent on N(9). Indeed, the molar absorptivity of the 2-chloro-6-fluorotoluene moiety of arprinocid at 258 nm is less than 2% of that of the intact drug. The absorption spectrum of Figure 1 is nearly superimposable on the absorption spectrum of an equimolar mixture in 0.1N HCl of adenosine [chosen to represent the N(9) substituted adenine] and of 2-chloro-6-fluorotoluene.

Comparison of Methods.—The accuracy of the determination of arprinocid in premixes over the concentration range 10–14% by HPLC analysis has been demonstrated previously (3). Table 6 presents a comparison of analytical results ob-

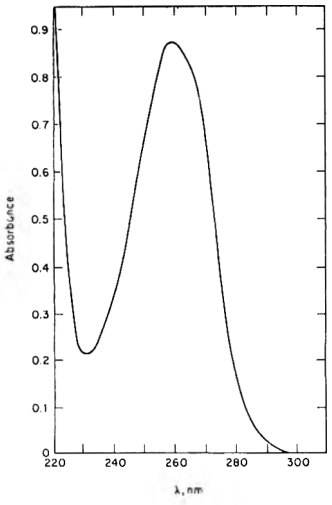

Figure 6. Absorption spectrum of arprinocid. Solvent: 0.1N HCl. Concentration 5.58 × 10^{-5}M.

tained by the present direct spectrophotometric procedure and by adsorption HPLC on a silica column with a mixed solvent mobile phase of methanol–water–chloroform (3 + 0.2 + 97). Each premix was analyzed in duplicate by both methods by one analytical chemist. The relative difference between the means of the 2 methods ranged from 0 to 5% and averaged 2%, demonstrating comparable accuracy and close agreement between the 2 methods over a wide range of concentrations.

Comments of Collaborators

That no comments were received concerning the practice samples would suggest that the participants were satisfied with their results on both the known and unknown practice samples and that they had no suggestions for improving the method at that time. Two collaborators used a sonic bath to aid dissolution of the drug into 0.1N HCl in the presence of CCl_4. Although the collaborative method included a precaution for additional mechanical shaking time at this step to ensure quantitative dissolution, the ultrasonic treatment is an excellent suggestion which is included in the method.

Conclusions and Recommendation

The 16 participating laboratories in this study present a random sampling of the application of this method on 13 different spectrophotometers—which include models manufactured by the Varian (Cary), Perkin-Elmer, Pye Unicam, and Beckman Instruments companies, and also include one flow-through cell—to demonstrate the applicability of the method on a wide range of instruments. The mean molar absorptivity at 258 nm from 44 total measurements of arprinocid reference standards reported by 13 of the collaborators (exclusive of Laboratories 1, 2, and 3) was 1.59 × 10^4 L/mole-cm, with a coefficient of variation of 2.3%. All the absorbance values reported by Collaborator 3 were 2.5–2.7 times the values found by all other collaborators, indicating either that this collaborator did not carry out the dilution pattern for both standards and samples exactly as prescribed in the method, or that the spectrophotometer was producing erroneously high absorbance values. At any rate, this collaborator was not excluded from the statistical analysis because it is apparent that the general principles of the method were nevertheless retained. The low results from this collaborator (Table 3) were not surprising in light of the high absorbances (>1.00) measured for both samples and standards.

Table 6. Comparison of spectrophotometric and HPLC methods (% arprinocid found)

	Spectrophotometric		HPLC		Diff. between
Sample	1	2	1	2	means
A	10.3	10.3	10.2	10.3	0.0
B	10.2	10.3	10.1	10.2	0.1
C	12.1	12.0	12.0	12.0	0.1
D	12.3	12.3	12.1	12.1	0.2
E	14.2	14.6	14.1	14.1	0.3
F	14.2	14.6	14.0	14.0	0.4
G	16.1	16.5	15.9	15.9	0.4
H	16.4	16.4	15.7	15.7	0.7

Although the increasing trend of random error with drug level which is underscored by Figure 5 has not been accounted for, its small absolute value can be tolerated in the method. It is also significant that the s_r^2 line on Figure 5 passes through its minimum in the concentration range of the complete formulated commercial premix product. This method has also been applied to aged premixes after storage for 1 year at 30°C with unchanged analytical results. The statistical analyses of this study were performed according to the *Statistical Manual of the AOAC* after elimination of 1 outlying laboratory and 8 individual outlying results. Inclusion of all the collaborative data of Table 2 in the computations, however, does not markedly alter the results. Although recomputation including all the outliers does increase the overall coefficient of variation of the data in Figure 3 to 5.5%, the

mean, median, and mode of the resulting distribution are 100, 99.3, and 100% recovery, respectively. Based on these collaborative results, it is recommended that the method be adopted official first action.

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References

(1) Fink, D. W., Martin, R. P., Pivnichny, J. V., & Shim, J.-S. K. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1078–1082

(2) Fink, D. W., Fox, A., & Pivnichny, J. V. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 1–4

(3) Fink, D. W. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 973–979

(4) Stong, J. D., & Fink, D. W. *Analyst*, in the press

(5) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, Va

(6) Library software program RANUM, MARK III, Information Services Division, General Electric Co.

(7) Laitinen, H. A. (1960) *Chemical Analysis*, McGraw-Hill, New York, NY, p. 555

High Pressure Liquid Chromatographic Determination of Supplemental Methionine in Poultry Premix

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Supplemental methionine was extracted from a feed sample with 0.1M HCl and separated by reverse phase or ion-exchange high pressure liquid chromatography and isocratic elution with KH_2PO_4 buffer solution as the mobile phase. Methionine was detected at 205 nm. The most reliable results were obtained by using reverse phase chromatography, 0.05M KH_2PO_4 buffer at pH 2.6 as mobile phase, and tyrosine as internal standard.

The amino acid methionine is an essential constituent of animal nutrition (1, 2) and should be taken into consideration in manufacturing feed rations, especially for young animals. It would be very expensive and uneconomical to meet the methionine demand only from high protein substrates. Therefore, synthetic methionine has become an important nutritional supplement in manufactured poultry feed (3) and premix (vitamins–minerals–antibiotics feed supplements).

Several methods are used for determining amino acids: gas chromatography (4), microbiological techniques (5), and liquid chromatography (amino analyzers and high pressure liquid chromatography (HPLC)). Protein hydrolysis by 6N HCl before determination is common to all these methods; treatment of the hydrolysates depends on the method used.

Liquid chromatographic methods are based on ion-exchange separation, but derivatization with dansyl chloride (6, 7), or fluorescamine (8) is necessary for the fluorometric detection of free amino acids. For spectrophotometric detection, addition of ninhydrin is required (9–11), as shown by Fahnenstick and Tanner (12), who determined supplemental amino acids in feed.

This paper describes a rapid, quantitative method for determining supplemental methionine in a premix. The method involves a simple extraction with 0.1M HCl followed by HPLC separation and direct measurement at 205 nm, thus avoiding conversion to a fluorometrically or colorimetrically detectable compound. Our method is therefore simpler and less time-consuming than other known methods.

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METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Perkin-Elmer Model 601 with Rheodyne loop injector. Operating conditions: wavelength 205 nm, pressure 400 psi, flow rate 1 mL/min, temperature ambient, sensitivity 0.02 AUFS, chart speed 30 in./h, mobile phase 0.1, 0.05, or 0.01M KH_2PO_4 adjusted to pH 2.6, 3.2, or 3.8.

(b) *Detector.*—UV-VIS continuously variable wavelength absorption spectrophotometer, Perkin-Elmer Model LC 55.

(c) *Recorder.*—Perkin-Elmer Model 123; calculating integrator, Perkin-Elmer Model M-2.

(d) *HPLC columns.*—LiChrosorb RP-18 (Knauer KG, BRD), 25 cm × 4.6 mm id. Partisil-10 SCX (Whatman, Inc., Clifton, NJ 07014), 25 cm × 4.6 mm id.

(e) *Standard solutions.*—Methionine (1000 ppm) and tyrosine (500 ppm) (Serva Feinbiochemica, BRD) in 0.05M phosphate buffer. Prepare working standards in buffer solution identical to mobile phase used.

(f) *Samples.*—Premix A was prepared according to standard recipe for poultry premixes. It contained all components except methionine (all vitamins, microelements, antibiotics, and pollard as carrier). Premix B was made in the same way, with addition of 100 ppm methionine.

Preparation of Samples

Weigh premix A into 3 laboratory bottles, 5 g per bottle. Add 40, 30, and 20 mg methionine, respectively, to make samples with 8000 (Sample A_1), 6000 (Sample A_2) and 4000 ppm methionine (Sample A_3). Add 30 mL 0.1M HCl to each sample and mix 15 min. Filter suspension through rough paper and evaporate extract under vacuum at 35°C. Wash residue in vacuum flask with 20 mL water and dry again. Dissolve sample in 10 mL 0.1M HCl and transfer to 100 mL graduated flask. Wash vacuum flask twice with 10 mL 0.1M HCl. Add 0.05M KH_2PO_4 phosphate buffer to volume.

Take 2 mL aliquot from Sample A_1, 4 mL from Sample A_2, and 5 mL from Sample A_3 and transfer to three 10 mL graduated flasks. Add 0.8 mL

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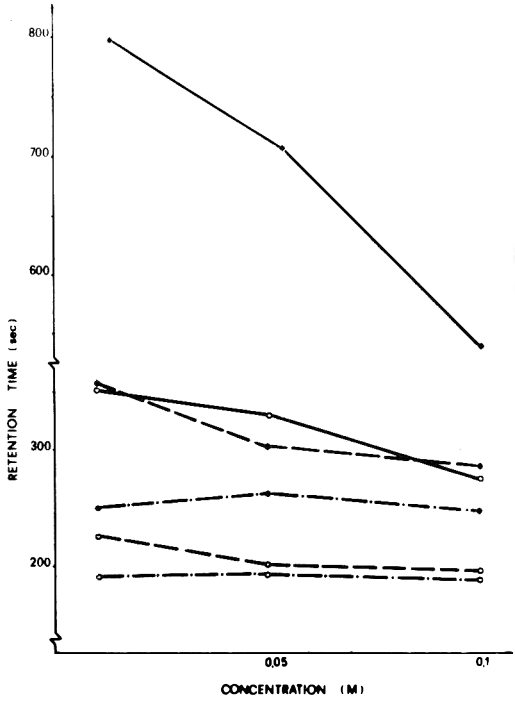

Figure 1. Influence of concentration and pH of mobile phase on retention time of methionine and tyrosine for reverse phase column: — pH = 2.6; – – pH = 3.2; -·- pH 3.8; O methionine; * tyrosine.

tyrosine standard solution to each flask and dilute with 0.05M phosphate buffer.

Weigh 10 g premix B and repeat procedure above, through "Wash vacuum flask twice with 10 mL 0.1N HCl." Add tyrosine standard solution and dilute to volume with 0.05M phosphate buffer.

Similarly, prepare sample solution without tyrosine. Filter all extracted preparations through 0.25 μm Millipore filter, and analyze by HPLC for methionine content.

Results and Discussion

To determine the parameters for separation of methionine, we used the fact that the ionic strength and pH of the eluant can influence the ion-exchange chromatographic separation of weak acids and alkalies (13). We used different concentrations and pH of buffer solutions to achieve a satisfactory separation. Both ion-exchange and reverse phase chromatography were done with the same samples and the same eluants.

Retention Time

Because the analysis involved 9 eluants of different concentrations and pH values and 2 chromatographic techniques for each amino acid

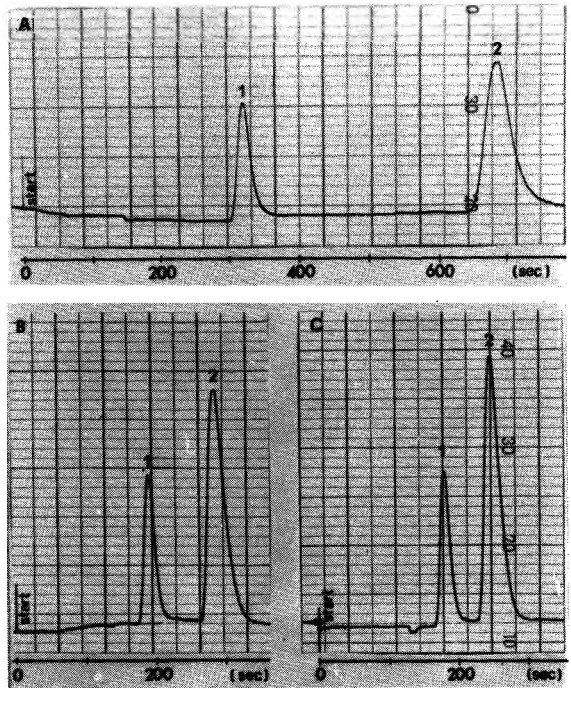

Figure 2. Changes in retention time of methionine (1) and tyrosine (2) according to pH of eluant: LiChrosorb RP-18; methionine + tyrosine (100 ppm + 40 ppm); 0.05M KH_2PO_4, (A) pH = 2.6, (B) pH = 3.2, (C) pH = 3.8.

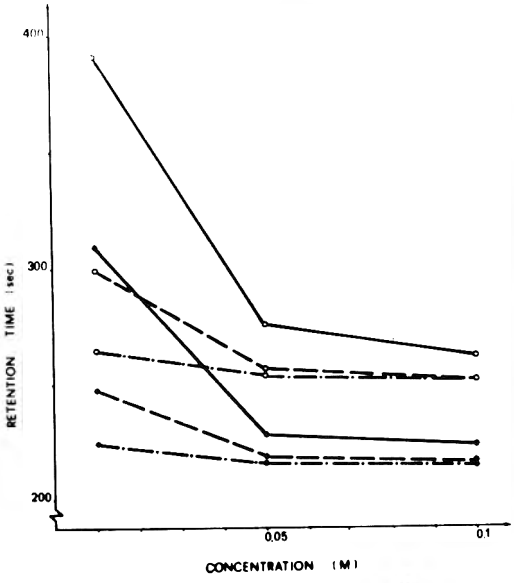

Figure 3. Influence of concentration and pH value of mobile phase on retention time of methionine and tyrosine for ion-exchange column: — pH = 2.6; – – pH = 3.2; –·– pH = 3.8; O methionine; * tyrosine.

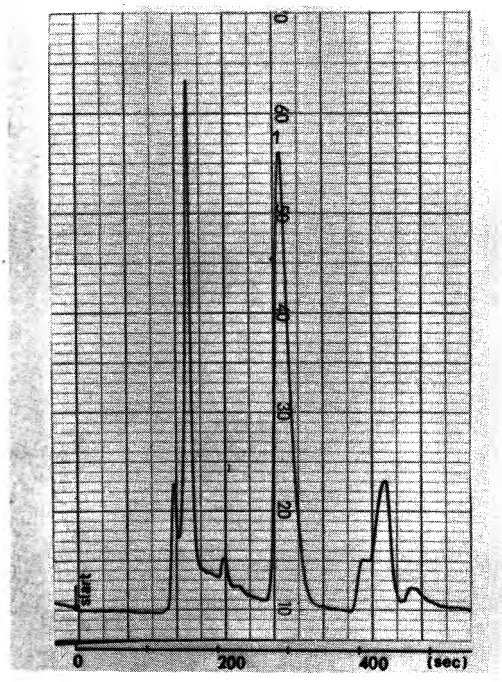

Figure 4. Chromatogram of premix: Partisil-10 SCX; 0.01M KH_2PO_4; pH = 3.2; (1) methionine.

(methionine and tyrosine), 18 retention times were obtained. Each result was the arithmetic mean of 3 successive injections.

A comparison of ion-exchange and reverse phase chromatography showed that changes in retention times, depending on pH and concentration of mobile phase, were more pronounced with reverse phase chromatography: The retention time of methionine varied from 186 to 352 s, and of tyrosine from 247 to 793 s (Figure 1). Differences in retention times for methionine and tyrosine, which can also indicate efficiency of separation, ranged from 65 to 440 s. As pH of the eluant increased from 2.6 to 3.8, retention times for methionine and tyrosine decreased regardless of the concentration of the mobile phase (Figure 2A, B, C). Retention time was inversely correlated with pH value and eluant concentration, but the effect of the change in pH was more intense.

With ion-exchange chromatography, the situation was similar. By increasing pH and eluant concentration, retention times of methionine and tyrosine decreased, but to a smaller degree (Figure 3). There was almost no difference for either amino acid with 0.05 and 0.1M KH_2PO_4. Regardless of the pH value, differences in retention times between methionine and tyrosine at the above mentioned concentrations of the eluant were not greater than 48 s. This means that an interference with some component in the sample is possible. A somewhat greater difference (82 s) in retention times for tyrosine and methionine was achieved with 0.01M KH_2PO_4, at pH = 2.6, which can therefore be regarded as the most convenient mobile phase for ion-exchange chromatography.

Quantitative Measurement

The experiments with all 8 combinations of mobile phase on both columns were performed with a sample of premix B, which was prepared for injection with or without tyrosine as internal standard.

In ion-exchange chromatography, tyrosine eluted before methionine and at the same time as interferences in the sample, so it was not suitable as internal standard. Methionine was quantitated with the aid of an external standard at pH 2.6–3.8 with 0.01M KH_2PO_4 buffer solution (Figure 4).

The least efficient separation on the reverse phase column was at pH 3.2 (Figure 5B). If pH was increased to 3.8 with all 3 buffer concentrations, methionine was separated from the ballast peak which interfered at pH 3.2. Although peaks were not well separated (Figure 5C), precise results could be obtained by means of an integrator. The most reliable and reproducible

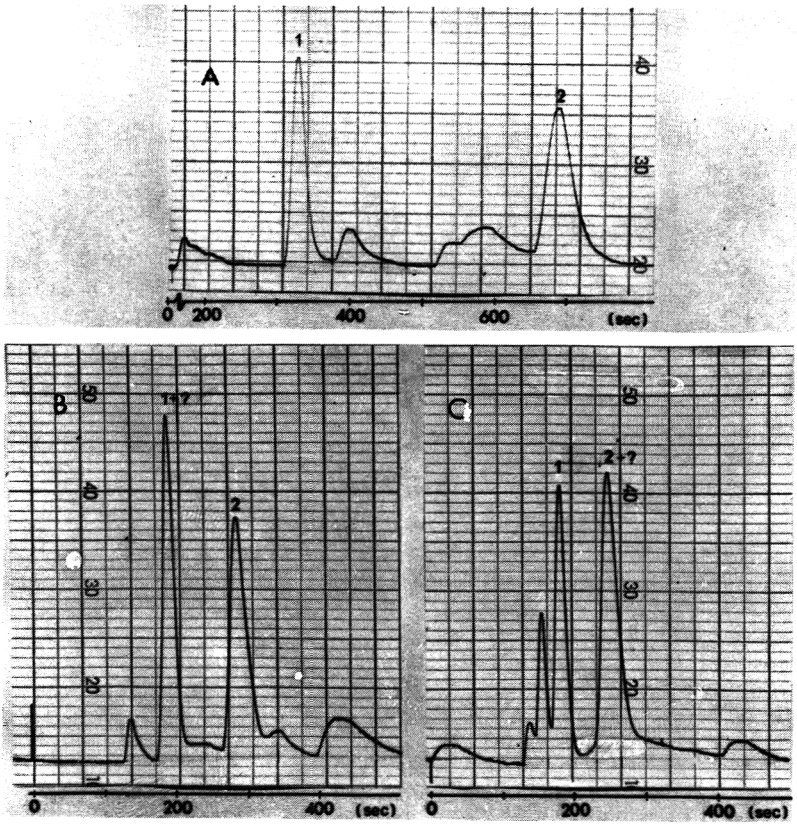

Figure 5. Chromatogram of premix with addition of tyrosine (40 ppm) as internal standard: LiChrosorb RP-18; 0.05M KH_2PO_4; (A) pH = 2.6; (B) pH = 3.2; (C) pH = 3.8; (1) methionine; (2) tyrosine.

results were achieved with 0.05M KH_2PO_4 at pH 2.6 (Figure 5A), because the difference in retention times was good (370 s), and there was no interference.

We analyzed 7 samples containing 100 ppm methionine (premix B) and 3 groups of 5 samples each, with 4000, 6000, and 8000 ppm methionine (premix A). Tyrosine was added to all samples as internal standard. Average results for 100, 4000, 6000, and 8000 ppm methionine were 98.10, 1.35% CV, 3904, 0.69% CV; 5971, 1.18% CV; and 7854, 0.99% CV, respectively.

REFERENCES

(1) Scott, M. L., Malden, C. N., & Young, R. J. (1969) in *Nutrition of the Chicken*, M. L. Scott et al. (Eds), Cornell University, Ithaca, NY, pp. 57, 96
(2) Haresign, W., Swan, H., & Lewis, D. (1977) *Nutrition and the Clinical Environment*, Butterworths, Boston, MA, p. 104
(3) Henkel, H. (1967) in *Syntetische Aminosäuern als Futtermittel in Handbuch der Futtermittel*, M. Becker, & K. Nehring (Eds) P. Parey Verlag, Hamburg-Berlin, GFR, p. 280
(4) Gehrke, C. W., Kuo, K., & Zumwalt, R. W. (1971) *J. Chromatogr.* **57**, 209
(5) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Arlington, VA, secs. **43.148-43.158**
(6) Smidt, G. J., Adams, R. F., Vandemark, F. L., & Slavin, W. (1977) *Chromatogr. Newsl.* **5**, 3, 33-36
(7) Bayer, E., Grom, E., Kalteneger, B., & Uhman, R. (1976) *Anal. Chem.* **48**, 8, 1106-1109
(8) Zeck, K., & Voelter, W. (1975) *Chromatographia* **8**, 7, 350-351
(9) Beck, A., Smidtborn, H., Spindler, M., & Tanner, H. (1978) *Kraftfutter* **3**, 118-124
(10) Heese, J., Jahn, G., & Fahnenstick, R. (1971) *Z. Tierphysiol. Tierernähr. Futtermittelkd.* **28**, 161-171
(11) Heese, J., Jahn, G., & Fahnenstick, R. (1972) *Z. Tierphysiol. Tierernaehr. Futtermittelkd.* **28**, 307-320
(12) Fahnenstick, R., & Tanner, H. (1974) *Landwirtsch. Forsch.* **27**, 1, 46-50
(13) Engelhardt, H. (1977) *Hochdruckflüssigkeitschromatographie*, 2nd Ed., Springer Verlag, Berlin, GFR, pp. 206-209

DRUG RESIDUES IN ANIMAL TISSUES

Confirmatory Identification of Carbadox-Related Residues in Swine Liver by Gas-Liquid Chromatography/Mass Spectrometry with Selected Ion Monitoring

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A confirmatory method has been developed to identify quinoxaline-2-carboxylic acid, the carbadox tissue residue, in swine liver, a target tissue, at the regulatory level of 30 μg/kg. Quinoxaline-2-carboxylic acid (QCA) is isolated from liver hydrolysates by solvent extraction and ion-exclusion chromatography, and a methyl ester derivative (CP-25,536 or QME) is identified by gas-liquid chromatography/mass spectrometry with selected ion monitoring. The relative intensities of 3 ions: the base peak at m/z = 130, a second significant mass at m/z = 158, and the molecular ion (M^+) at m/z = 188, are monitored simultaneously with a quadrupole mass spectrometer. Validation studies consisting of the analysis of liver fortified with QCA at the regulatory level and analysis of swine specimens containing physiologically incurred carbadox residues demonstrated that peak height ratios of ions in these tissue extracts corresponded to ion intensities of standards monitored at m/z = 188, 158, and 130.

Carbadox [hydrazinecarboxylic acid (2-quinoxalinylmethylene)-, methyl ester N^1,N^4-dioxide] is used as a growth-promoting and chemotherapeutic agent for treatment of enteric diseases in young swine. It is the active compound in Mecadox, a medicated premix, for use in swine starter feeds at a level of 0.0055%. To ensure absence of detectable residues of carbadox and safety of meat products to the food consumer, the drug is withdrawn from swine several weeks before they reach market weight (1).

The assay and depletion of residues of carbadox have been investigated in pigs by radiotracer metabolism studies. Carbadox is rapidly metabolized to several compounds. These residues are higher and more persistent in liver than in kidney, considerably lower in muscle, and nondetectable in fat. Quinoxaline-2-carboxylic acid (QCA) has been identified in tissue hydrolysates as the last remaining major residue, and is designated the marker substance (2).

The official carbadox tissue residue assay measures QCA in hydrolysates of tissue by electron capture gas-liquid chromatography (GLC-ECD) of an *n*-propyl ester derivative (1). The depletion of QCA from tissues assures absence of its precursors, i.e., drug and its initial metabolites. Because liver is the target tissue, i.e., metabolite residues are higher and more persistent in liver than in other tissues, chemical analysis for QCA in liver is sufficient to detect misuse of the drug. The regulatory level for QCA in swine tissue is 30 μg/kg.

Chemical assay residue depletion studies with the official method for QCA have shown that 28 days are required for depletion of carbadox residues below 30 μg/kg in swine liver. Essentially the same results were found by Skarka et al. (3) and Sestakova et al. (4) who used polarographic methods to analyze tissues of swine and calves fed carbadox.

GLC-ECD or polarographic analytical methods do not unequivocally confirm the identity of QCA. For this reason, we used combined gas chromatography/mass spectrometry with selected ion monitoring, which has been recommended by others (5–9) for confirmatory identification of residues in tissues. Also, to simplify the recovery and derivatization of QCA, we developed alternative isolation and esterification procedures. The application of this confirmatory method to the analysis of control, fortified, and withdrawal swine liver specimens is the subject of this report.

METHOD

Principle

Alkaline hydrolysis of swine liver liberates quinoxaline-2-carboxylic acid as a major metabolite of carbadox. This acid is separated from major interfering tissue components by sequential extraction into ethyl acetate and pH 6.0 buffer followed by an ion-exclusion chromatography step. The column eluate is extracted with $CHCl_3$, concentrated by evaporation of the solvent, and then esterified with methanolic

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sulfuric acid to convert it to a readily extractable methyl ester derivative (QME or CP-25,536) that is suitable for analysis by gas-liquid chromatography/mass spectrometry.

Reagents

All solvents were glass distilled, or equivalent. No special precautions were taken with glassware. All chemicals were reagent grade.

(a) *Ion-exclusion resin.*—Macroporous, strong acid cation exchanger, AG MP-50, 100–200 mesh (Bio-Rad Laboratories, Richmond, CA 94804).

(b) *Analytical standards.*—QCA (CP-16,505), and QME (CP-25,536) (provided by Central Research, Pfizer Inc., Groton, CT 06340).

(c) *Citric acid buffer.*—0.5M. Adjust pH of 100 mL 1M citric acid to pH 6.0 with 5M NaOH (ca 55 mL), using previously calibrated pH meter. Adjust final volume to 200 mL with water. (Note: Before making final pH adjustment, cool buffer to room temperature.)

(d) *Methanol–sulfuric acid reagent.*—97 + 3. Dilute 3.0 mL sulfuric acid to 100 mL with methanol previously dried over anhydrous Na_2SO_4 (prepare daily and use ice bath).

Preparation of Standard Solutions

(a) *QCA.*—Weigh exactly 1.50 mg analytical standard and dissolve in 100 mL methanol (stock solution). Dilute 1.0 mL stock solution to 100 mL with methanol (solution A). Also dilute 1.0 mL of stock solution to 100 mL with water (solution B).

(b) *QME.*—Weigh exactly 1.50 mg analytical standard and dissolve in 100 mL of methanol (stock solution). Dilute 1.0 mL stock solution to 100 mL with toluene.

Apparatus

(a) *Mass spectrometer.*—Finnigan Model 3200 quadrupole instrument equipped with Promim unit for monitoring selected ions was used in conjunction with Model 9500 gas chromatograph. Glass GC column, 1 m × 2 mm id, packed with 3% Silar 10C on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories). Before use, packed column was conditioned overnight at 250°C with low gas flow. Helium was used as carrier gas (10 lb/in.^{2}). Vacuum diverter was used to vent column effluent. Injector, column oven, separator oven, and transfer line were operated at 230, 190, 220, and 220°C, respectively. Under these conditions, retention time of QME was 1.25 min. Ionization energy was set at 70 eV, and emission current was 0.74 mA. Ion chromatograms were recorded on a multi-pen Houston recorder at chart speed of 1 cm/min.

(b) *Chromatographic columns.*—25 cm long × 10.5 mm id, equipped with Teflon stopcocks and 200 mL reservoir capacity (S.G.A. Scientific Inc., Bloomfield, NJ 07003; Cat. No. JC-1506).

Column Preparation

Preparation of the AG MP-50 resin.—Transfer 100 g AG MP-50 resin (Bio-Rad Laboratories) to Buchner funnel equipped with fritted disc (coarse) and filter flask. Wash resin in sequence with 1 L methanol, 1 L water, and 500 mL 1N HCl. Stir resin with glass rod and maintain a moderate filtration rate by using suction. Store filter cake in capped amber jar.

Preparation of ion-exclusion columns.—Mix ca 7.0 g washed AG MP-50 resin in 1N HCl and transfer to 10.5 mm id glass column containing a small glass wool plug to retain the resin. Pack resin to height of 4 in., using glass rod, and cap resin bed with glass wool plug. Maintain level of 1N HCl slightly above resin bed.

Procedure

Dissolution and hydrolysis.—Transfer 5.0 g freshly sliced frozen tissue to 50 mL centrifuge tube. Pipet 10.0 mL 3M NaOH into tube, stopper lightly, and place it in pre-heated silicone oil bath set at 95–100°C for 30 min. (Note: Level of silicone oil bath should exceed that of tissue sample.) To validate detection of carbadox residue at regulatory level, fortify 5 g tissue with 150 ng quinoxaline-2-carboxylic acid or 1 mL QCA working standard solution B. Add alkali and proceed as directed above.

Extraction of hydrolysate.—Cool alkaline hydrolysate in ice bath and acidify to ≤pH 1 (deep red to alkacid test paper) with 4 mL HCl. Add 15 mL ethyl acetate to acidified hydrolysate, stopper, and extract by shaking 20 s. Centrifuge mixture 5 min at 1500 rpm to clarify ethyl acetate phase. Recover ethyl acetate phase with blow-out pipet equipped with pro-pipet bulb, and transfer extract to 60 mL separatory funnel equipped with Teflon stopcocks. Re-extract hydrolysate with 2 additional 15 mL portions of ethyl acetate and combine organic extracts. (Caution: Do not contaminate ethyl acetate phase with interfacial material during these extractions.)

Add 5 mL 0.5M pH 6.0 citric acid buffer to ethyl acetate extract, shake, and let lower phase clarify (ca 10 min). Collect aqueous phase in 50 mL glass-stopper centrifuge tube. Re-extract

ethyl acetate phase with additional 5 mL pH 6 buffer, wait for aqueous phase to clarify, and combine aqueous extracts with 2 mL HCl.

Ion-exclusion chromatography.—Transfer acidified aqueous extract to ion-exclusion column prepared as described above. Drain extract to top of resin bed. Wash tube and resin with 20 mL 1N HCl and drain wash through column. Rewash column with additional 20 mL 1N HCl. Discard this and previous effluents from column. Place 250 mL separatory funnel under column and elute column with 75 mL 10% methanol in water. Column may run dry in this step. (Note: Flow rate of effluent is ca 1.2 mL/min. This resin has been discarded after each assay, but may be regenerated by washing in sequence with methanol, water, and 1N HCl.)

Concentration of quinoxaline-2-carboxylic acid eluate.—Add 1.0 mL HCl to eluate and extract with three 50 mL portions of $CHCl_3$. Collect extracts in 250 mL round-bottom flask and evaporate to dryness on rotary evaporator at 45–50°C. Transfer residue to 15 mL centrifuge tube by washing flask with 3 small portions (ca 1.0 mL each) of methanol. Place tube in 55°C water bath and evaporate solvent to dryness under stream of nitrogen. Esterify this residue as indicated below. (Note: Use disposable Pasteur pipet to transfer methanolic residue.)

Esterification of quinoxaline-2-carboxylic acid.—Reconstitute residue with 0.2 mL freshly prepared (97 + 3) methanol–sulfuric acid. Stopper and heat 30 min in 50–55°C water bath. Remove tube from water bath, add 0.1 mL toluene to tepid esterification solution, and thoroughly mix on test tube mixer. Follow with addition of 1.0 mL water, mix thoroughly, and centrifuge to clarify. With aid of disposable Pasteur pipet, remove most of aqueous layer. Process toluene extract by gas-liquid chromatography/mass spectrometry with selected ion monitoring (GLC/MS-SIM). (Note: Toluene extracts are stable for at least 3 months when stored at reduced temperature to prevent losses through evaporation.)

Preparation of process standards for GLC/MS-SIM analysis.—Pipet 1.0 mL working standard solution A of quinoxaline-2-carboxylic acid (0.150 μg/mL) into 15 mL centrifuge tube and evaporate to dryness under stream of nitrogen at 55°C. Add 0.2 mL methanol–sulfuric acid (97 + 3), stopper, and incubate 30 min at 55°C. Extract as directed above (*Esterification of quinoxaline-2-carboxylic acid*).

Gas-liquid chromatography/mass spectrometry.—Set mass spectrometer to detect ion current profiles at m/z = 188, 158, and 130 via Promim units. Inject 6–12 ng preformed CP-25,536 (QME) (1.50 μg/mL toluene) into gas chromatograph to determine its retention time and to evaluate response of GLC/MS detector. Adjust optimum ion source voltages for maximum sensitivity consistent with good peak shape. Following this tuning procedure, injection of neat toluene should give no response at ions of interest. Follow this with μL injections of process standard, control, and sample tissue extracts. Allow 10 min between sample injections to clear gas chromatograph of background peaks. Measure peak height (mm) of samples and standards for each ion current profile. Normalize results with respect to intensity of base peak at m/z = 130.

Results and Discussion

Studies were conducted to optimize recovery and isolation of QCA from co-extractives of tissue hydrolysates, its conversion to a derivative suitable for gas-liquid chromatography, and analysis by mass spectrometry. Earlier studies showed that an extended hydrolysis of liver is not required to liberate QCA (2). Its recovery is controlled by the efficiency of the isolation steps.

QCA is quantitatively extracted into ethyl acetate or chloroform from strongly acidic (pH ≤1) solutions, and is readily extracted into aqueous buffered solutions at pH 6 or higher because it is a strong carboxylic acid (pKa 2.88) (10). The partitioning of QCA into ethyl acetate and then pH 6 buffer isolates it from a complex mixture of tissue hydrolysates, and provides an aqueous extract that is suitable for purification by ion-exclusion chromatography.

The ion-exclusion process (11) is useful because it provides the means for trace collection of QCA and its separation from non-electrolytes. Like other aromatic and aliphatic carboxylic acids, QCA in its non-ionized form (pH ≤1) is sorbed by the matrix of the resin polymer and is excluded as the dissociated species. Several examples of this technique have been reported for organic acids (12–16). In the application described here, an organic modifier, methanol, is added to the eluting solvent to expedite the recovery of QCA. Among strong cation exchange resins examined, the macroporous resin AG MP-50 was selected because of its non-swelling properties and its apparently greater sorptive capacity for non-electrolytes. Under the recommended use conditions, 150 ng QCA, or the equivalent amount of compound required for assay at the regulatory level, is recovered in the ion-exclusion chromatographic and subsequent

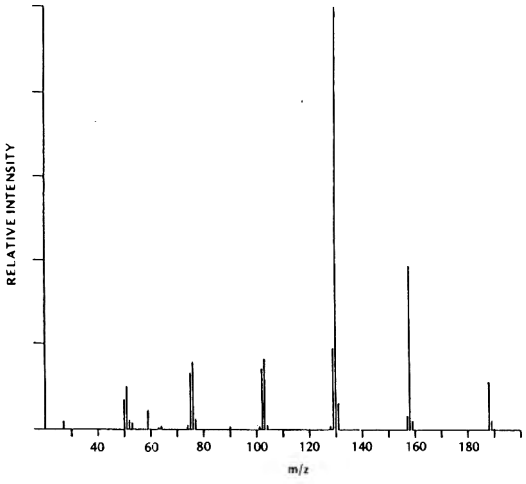

Figure 1. Normalized EI mass spectrum of QME.

extraction steps with an efficiency of 82 ± 6.7% (n = 10).

Among several esterification procedures examined (17), sulfuric acid catalysis with dried methanol provided the best yields, convenience, ease of extraction, and compatibility with the GLC/MS system. The esterification of QCA to QME is complete under the recommended conditions for derivatization. The facile extractability of QME into small volumes of toluene or benzene from the derivatization medium also provides a simple method for concentrating the sample.

The mass spectrum of QME under electron impact (EI) ionization conditions at 70 eV, as presented in Figure 1, is characterized by prominent ions above m/z 100 at 188, 158, 130, 129, 103, and 102. Among these ions the molecular ion at m/z 188, the base peak at m/z 130, and a third significant ion at m/z 158 were chosen for selected ion monitoring. These ions were chosen from the EI spectrum because they are more diagnostic for QME, and are less subject to interference by co-eluting compounds. No fragmentation of the M + 1 ion (189) was observed under chemical ionization (methane) conditions and, therefore, this technique provided no ion ratios to further confirm the presence of QCA.

For this application of the selected ion monitoring technique, peak ion ratios were determined with a quadrupole mass spectrometer (18). This instrument with its ability to simultaneously

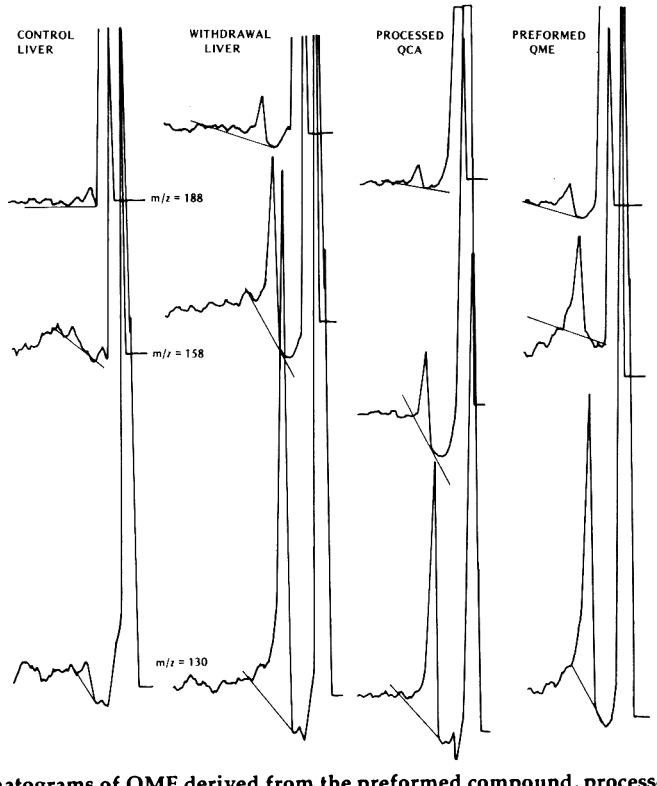

Figure 2. Ion chromatograms of QME derived from the preformed compound, processed QCA, and withdrawal swine liver. Pens were offset to monitor ions at m/z = 188, 158, and 130.

Table 1. GLC/MS-SIM analysis of fortified and withdrawal liver samples for carbadox-related residues, 2 trials

Standard-sample identity	No. of replicates	Intensity ratios, 188/158/130		
Trial 1				
Preformed QME	3	11	33	100
QCA converted to QME (process stds)	3	8	32	100
Swine liver[a] fortified with QCA at 30 μg/kg	2	9	33	100
Withdrawal liver contg 80 μg/kg of QCA (assayed by 21 CFR 556.100)	6	9	32	100
Trial 2				
Preformed QME	3	15	27	100
QCA converted to QME (process stds)	3	16	27	100
Swine liver[a] fortified with QCA at 30 μg/kg	3	14	26	100
Withdrawal liver contg 30 μg/kg of QCA (assayed by 21 CFR 556.100)	6	12	26	100

[a] No carbadox-related residues were detected in control liver.

monitor any 3 ions in the mass spectrum of QME has an advantage over many magnetic sector mass spectrometers which have a limited range for SIM. In this particular case a magnetic sector mass spectrometer with a limitation in mass range of approximately 10% can acquire only one ion current profile at a time (e.g., m/z 130). This means that the ion current profiles for blanks, standards, and samples cannot be acquired simultaneously, but must be monitored sequentially, a process which makes the assay cumbersome and less precise.

The procedure described here was evaluated by assaying control liver specimens, tissue fortified with QCA at the regulatory level (30 μg/kg), and withdrawal specimens containing incurred residues at 30 and 80 μg/kg. Two trials were conducted and representative chromatograms are presented in Figure 2. These analyses (Table 1) demonstrated that within each trial, 1 or 2, peak height ratios for the fortified and withdrawal samples corresponded to ion intensities of the processed and preformed standards. No major or significant interference was found in extracts of control liver.

Based on these results, the GLC/MS-SIM procedure is suitable for verifying the identity of carbadox-related residues in swine liver at and above the regulatory level.

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References

(1) *Fed. Regist.* (October 3, 1972) 37 FR 20683; CFR, § 556.100
(2) Ferrando, R., Truhaut, R., Raynaud, J. P., & Spanoghe, J. P. (1975) *Toxicology* **3,** 369–398
(3) Skarka, P., Sestakova, I., & Frgalova, K. (1976) *Biol. Chem. Vyz. Zvirat* **12,** 209–213; *Chem. Abstr.* (1977) **86,** 104818q
(4) Sestakova, I., Skarka, P., & Manousek, O. (1980) *Biol. Chem. Vet.* **16** (22), 29–32; *Chem. Abstr.* (1980) **93,** 93648a
(5) Cala, P. C., et al. (1972) *J. Agric. Food Chem.* **20,** 337–340
(6) Day, E. W., Jr., Varratta, L. E., & Sieck, R. F. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 520–524
(7) Ryan, J. J., & Pilon J.-C. (1976) *J. Assoc. Off. Anal. Chem.* **59,** 817–820
(8) VandenHeuvel, W. J. A., III, Wood, J. A., DiGiovanni, M., & Walker, R. W. (1977) *J. Agric. Food Chem.* **25,** 386–389
(9) Stan, H.-J., & Abraham, B. (1980) *J. Chromatogr.* **195,** 231–241
(10) Gum, W. F., Jr., & Joullie, M. M. (1965) *J. Org. Chem.* **30,** 3982–3985
(11) Wheaton, R. M., & Bauman, W. C. (1953) *Ind. Eng. Chem.* **45,** 228–233
(12) Harlow, G. A., & Morman, D. H. (1964) *Anal. Chem.* **36,** 2438–2442
(13) Goodman G. W., Lewis, B. C., & Taylor A. F. (1969) *Talanta* **16,** 807–812
(14) Lehotay J., & Traiter, M. (1974) *J. Chromatogr.* **91,** 261–266
(15) Hanai, T., Walton, H. F., Navratil, J. D., & Warren, D. (1978) *J. Chromatogr.* **155,** 261–271
(16) Tanaka, K., Ishizuka, T., & Sunahara, H. (1979) *J. Chromatogr.* **174,** 153–157
(17) Nicholson, J. D. (1978) *Analyst* **103,** 1–28
(18) Falkner, F. C., Sweetman, B. J., & Watson, J. T. (1975) *Appl. Spectrosc. Rev.* **10,** 51–116

DECOMPOSITION IN FOODS

Effect of Adsorption of Histamine to Glass Surfaces on Its Estimation

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Histamine is rapidly adsorbed on the surface of glass containers when stored in aqueous or 10% methanol solution at ambient or sub-zero temperatures. This effect can lead to marked errors in the determination of histamine. Addition of acid to the holding medium or storage in polyethylene containers eliminates this effect.

The consumption of fish such as mackerel is occasionally accompanied by scombrotoxic poisoning. It has been speculated that a relationship exists between the amount of histamine produced by spoilage organisms and the toxicity of the fish. The validity of this hypothesis is the subject of a current investigation (1) which requires the rapid analysis of large numbers of extracts from mackerel samples originating during an outbreak.

A variety of methods for estimating histamine have been described in the literature (2). One extremely sensitive technique, which is capable of measuring 10 ng of histamine, is based on its condensation with orthophthaldehyde (OPT) to form a stable fluorescent product. The analysis of extracts from biological tissues such as fish, however, is complicated by interference from histidine and to a lesser extent from other substances (3).

Systems developed for the removal of histadine have specified ion exchange (4, 5) or selective extraction techniques (6). Certain irregularities became apparent during modification of an automated system based on selective extraction (7), which uses a Technicon AutoAnalyzer coupled to a fluorometer. Histamine standards in 10% methanol solution, ranging in concentration from 50 to 400 ng/mL and stored in glass liquid scintillation counting vials for convenience, gave response variations that were a function of the storage time between preparation and assay.

It has been reported that histamine is more stable in polypropylene containers than in glass, and that under certain conditions it can become unstable in neutral or basic aqueous solution (2). In contrast with this finding, it is reported (8) that storage in 0.1N HCl in glass for 1 day at −20°C leads to a loss of 35% in fluorometric response, although the effect was not noted when polyethylene bottles were used. Adsorption of histamine to glass test tubes and powdered glass from aqueous solutions (10^{-6}–10^{-8}M) has also been noted (9), but no indication was given of the time such solutions were held before assay. In view of these different findings, the effects of storing histamine under various conditions in both glass and polyethylene containers have been investigated.

Experimental

Glass and polyethylene liquid scintillation counting vials were purchased from 2 different suppliers. The [ring 2 ^{14}C] histamine dihydrochloride (59.7 m Ci/m mol), [2,5 ^{3}H] histamine dihydrochloride (53 Ci/m mol), and carboxyl [^{14}C] histidine (50 m Ci/m mol) were purchased from Radiochemical Centre, Amersham, UK. The radiochemical purity of these materials (>96%) was checked by radio thin layer chromatography (RTLC) with a Panax thin layer scanner following chromatography on silica gel plates, using $CHCl_3$–methanol–17% NH_4OH (2 + 2 + 1). After scanning, spots were located by using a ninhydrin spray. Nonradioactive stock solutions of histamine and histidine were prepared and stored in polyethylene containers.

Adsorption of histamine and histidine to glass and polyethylene was studied by dispensing diluted portions of the radioactive materials as supplied into polyethylene vials containing 19 mL of either water or 10% methanol. Hand-operated pipets with polyethylene tips were used to dispense and remove aliquots of all solutions to avoid possible adsorption to measuring glassware. After removal of a zero time sample, contents of a number of vials were poured into empty glass vials before further assay of 1 mL portions. Samples for counting were placed in glass vials that contained 0.2 mL of a 10 mg/mL aqueous solution of the relevant carrier before

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Table 1. Activity of [^{14}C] histamine in 10% methanol solution after storage in glass and polyethylene vials

Vial type	Time held at 20°C				
	0, h	2, h	4, h	6, h	23, h
Glass	28 321[a]	14 621	7 786	5 883	2 591
Polyethylene	28 164	28 516	28 479	28 069	28 158

[a] Values are average disintegrations per minute from 4 vials of each type at each sampling.

addition of 10 mL NE 260 scintillant (Nuclear Enterprises, Edinburgh, UK). Activity was determined with a Nuclear Chicago Isocap liquid scintillation spectrometer using the sample channels ratio (SCR) method for estimating counting efficiency.

The effect on adsorption of reducing the specific activity of histamine was investigated within glass vials in a similar manner by first adding various levels of nonradioactive histamine carrier followed by a standard aliquot of [^{14}C] histamine to polyethylene vials. Variation in the histamine adsorbed due to the presence of HCl and NaOH was monitored by incorporating these components at different concentrations into [^{14}C] histamine solutions, with the glass and polyethylene vials held at 20°C and −20°C between assays. This experiment was repeated in glass vials at 20°C, using NaCl and histidine as replacements for the acid and base. Adsorption in the presence of 2 different scintillants was noted by introducing 0.2 mL volumes of [^{14}C] histamine (150 ng) into glass vials already containing 10 mL NE 260 or a similar volume of toluene–2-ethoxyethanol (T–2EE) (7 + 3) (10). In certain trials, 6 mL only of an aqueous [^{14}C] histamine solution was carefully placed on the bottom of glass vials and held for 20 h at 20°C. Initial and final activities were estimated and the vial contents were removed by using a Pasteur pipet attached to a vacuum line. Activity remaining within the vials was determined using external standard channels ratio (ESCR) and SCR techniques, after addition of 12 mL T–2EE scintillant, before and after introduction of 0.2 mL carrier histamine solution. The walls of other vials, after removal of aqueous phase as above, were thoroughly wetted with 0.5 mL diluted carrier and the solution was freeze-dried. Aliquots of the concentrated solution were subjected to RTLC. Solutions of histamine held in 0.1N HCl for 15 days at −20°C were similarly concentrated and chromatographed after removal from the glass vials used for storage.

The influence on histamine solutions of storage in a range of different containers including liquid scintillation counting vials and in soda and Pyrex glass test tubes was routinely monitored using [^{14}C] histamine. Occasionally, however, [^{3}H] histamine, because of its higher specific activity, was used as a more sensitive adsorption indicator.

Results

Initial experimentation was designed to test the different capacities of glass and polyethylene vials to adsorb histamine from solution. Table 1 lists findings obtained when 20 mL [^{14}C] histamine at a concentration of 23.7 ng/mL 10% methanol (2.1×10^{-7}M) solution was stored for various times in each type of vial. Essentially identical results were obtained for aqueous solutions of histamine. A slight difference in uptake between the 2 batches of glass vials was noted, 1 lot giving on average 12% less adsorption than its counterpart, but no adsorption was evident for any of the polyethylene vials. Replacing histamine with [^{14}C] histidine at a concentration of 40.1 ng/mL (2.6×10^{-7}M) showed that the latter was not adsorbed to glass or polyethylene under the same conditions, 99.2% of the initial activity was recovered after 23 h.

The range of concentrations of standards normally used for estimating histamine by the OPT method is between 100 and 500 ng/mL (4, 7). The extent of adsorption at concentrations of this order for 20 mL volumes of histamine held in glass vials is shown in Figure 1. Addition of HCl and NaOH at final concentrations of 0.0025–0.1M to aqueous histamine solutions (26.0 ng/mL) completely prevented loss of activity, at molarities of 0.01 or greater, in glass or polyethylene vials held at 20° or −20°C, whereas neutral solutions in glass lost activity at −20°C as expected. Vials held at 20°C were sampled at intervals up to 23 h, while those at −20°C were assayed less frequently over a period of 15 days. RTLC of samples stored in 0.1M HCl for 15 days indicated that 96% of activity (identical to that in the material as supplied) was associated with the carrier histamine spot.

In contrast with the marked effect of acid or

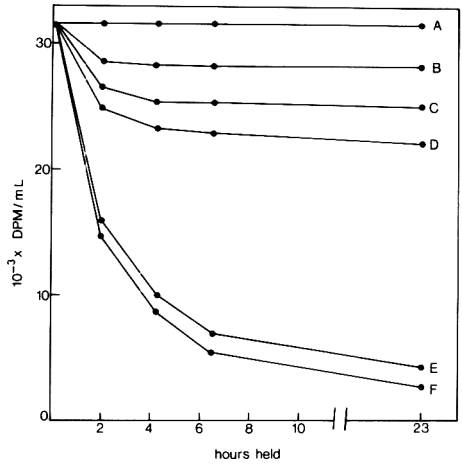

Figure 1. Change in the radioactivity of [^{14}C] histamine solutions (26.5 ng/mL) stored for varying times in glass vials in the presence of the following concentrations of carrier histamine: A, 1 mg/mL; B, 600 ng/mL; C, 400 ng/mL; D, 100 ng/mL; E, 50 ng/mL; F, 0 ng/mL. Results shown are average values/mL from 2 vials at each concentration.

base, greatly increased concentrations of NaCl were required to eliminate adsorption. To study this effect, various concentrations of salt were added to fixed portions of aqueous [^{14}C] histamine (18.4 ng/mL) held in polyethylene vials before transfer to glass vials. After 20 h at 20°C, [^{14}C] histamine in 0.005, 0.01, 0.025, 0.05, 0.1, and 0.25M NaCl solutions showed DPM values of 10.4, 19.5, 50.2, 71.2, 88.8, and 97.0, respectively, expressed as a percentage of initial activity/mL.

Histidine, as indicated earlier, although not itself adsorbed, protects more efficiently than does NaCl (Table 2). To avoid possible complications in interpreting the results, the free base rather than histidine hydrochloride was used to obtain the values shown in Table 2.

Interesting differences in the ability of the 2 scintillants to prevent adsorption of histamine emerged. When NE 260 was employed no adsorption was observed with [^{14}C] histamine held for 24 h in the absence or presence of carrier. Identical activities incorporated in T-2EE scintillant, however, in the absence of carrier gave, after holding for 1 and 24 h, respectively, only 83 and 72% of the activity observed using NE 260 or T-2EE with carrier. Estimation of activity remaining within vials that originally held 6 mL active histamine showed values using the SCR method that were 115% of those obtained from the same vials by the ESCR technique. The degradation of the [^{14}C] energy spectrum produced within the scintillant due to the close association of activity with the vial wall is responsible for this effect (11) and provides classic evidence of the existence of such adsorption (12). Addition of carrier histamine increased the count rate by about 44% initially, and calculated activities were similar by SCR and ESCR methods after holding for 72 h. The value obtained approximated that expected from a consideration of the initial and final solution activities during storage in the glass vial. Examination of adsorbed activity from similar vials demonstrated that 95% was coincident with the position occupied by authentic histamine, which strongly suggests that alteration of histamine did not occur during adsorption.

Discussion

The phenomenon of adsorption to glass surfaces, which can be effectively and elegantly demonstrated by the use of high specific activity radiochemicals, has been noted frequently for a variety of organic and inorganic materials (11-14). Highly sensitive analytical techniques, such as the OPT method for histamine, carry a potentially greater risk of error should this effect operate. Data in Table 1 show that histamine is quickly adsorbed to the surface of glass liquid scintillation counting vials such as those we previously used for storage of histamine standards, but it is not adsorbed on polyethylene containers. The fact that histidine is not adsorbed despite its structural similarity to histamine implies that the adsorption mechanism is not a direct consequence of the imidazole moiety, although other influences on structure may predominate in solution. From the dramatic effects of HCl and NaOH relative to those observed with NaCl it is obvious that $[H]^+$ and $[OH]^-$ operate to suppress adsorption. Various

Table 2. Effect of histidine on the adsorption of histamine to glass vials [a]

	Time held at 20°C	
Histidine, mM	3, h	20, h
0.033	43.1	6.7
0.066	40.3	7.5
0.166	45.9	16.6
0.333	51.2	20.6
0.666	56.3	27.0
1.000	56.9	33.3

[a] Values are expressed as a percentage of initial activity/mL. Each vial contained 29.4 ng histamine/mL with a histidine concentration as shown.

reasons can be advanced for these effects including deactivation of sites on the vial wall and alteration of the net charge on the histamine molecule.

Figure 1 demonstrates that the range of concentrations at which adsorption is apparent from 10% methanol solution exceeds that commonly used as standards by others in estimating histamine by OPT (4, 7). It should be stressed, however, that these authors used histamine solutions which were prepared, held, and diluted in relatively strong acid solution, with similar precautions taken at all stages of the estimation, a situation that we find completely prevents adsorption. Other workers (6) have apparently not found this precaution necessary when using 5 μM aqueous histamine as a reference solution, and no indication is given that such solutions should be acidified or stored in polyethylene containers. Of the large number of different types of glass containers examined for potential histamine adsorption, we have not found any that are free from the effect; some adsorb to a greater extent than do the liquid scintillation counting vials used for most experimental work here, although differences in magnitude between various glass types are apparent. Variations of about 15% between vials of the same type were also observed, presumably due to differences in the surface micro structure of individual containers.

In the AOAC method for the analysis of histamine in fish (4), methanol is used for extraction of the tissue and a comparison of different homogenizing media has shown this solvent to be superior to perchloric or trichloroacetic acid for its recovery from tuna (6). While it is likely that other constituents of the extract will exert a protective action on histamine adsorption similar to that noted with histidine (Table 2) and, as discussed above, to a lesser extent with NaCl, we recommend that methanol extracts be stored in polyethylene containers. Extracts stored in glass should be acidified. Holding neutral methanol extracts of cheese in glass containers may lead to a loss of histamine (15), and it may be significant that similar losses occurred (2) when using glass as opposed to polypropylene (4) ion-exchange columns. We suspect that the low absorbance of a histamine standard obtained by one of the analysts in a collaborative study of histamine in tuna samples (4) could have arisen from such a standard being prepared in aqueous rather than acid solution as recommended.

We are unable to explain the discrepancy between our results in which addition of HCl at concentrations of 0.01M or greater completely prevented the adsorption of histamine to glass vials and those of others (8) who find a considerable loss of fluorescence from solutions containing 1 mg/mL during storage in 0.1N HCl at −20°C for 1 day. The fact that 96% of activity is associated with the histamine spot on a TLC plate after storage in 0.1N HCl at −20°C for 15 days eliminates the possibility that degradation is a cause of the loss. It is also apparent that losses do not occur when acidic solutions of histamine are stored at refrigerator temperatures (4, 5).

The lack of adsorption of histamine to glass vials in the absence of carrier using NE 260 contrasts with the loss of activity noted in T-2EE. The former scintillant incorporates aqueous phase in a micellar form, whereas in the latter a true solution of the aqueous phase in the organic mixture exists. The observed differences are most likely to arise from the different modes of accommodation of the water component although, because the exact composition of the commercial scintillant is unavailable, other explanations cannot be dismissed.

The experimentation adopted in this work does not allow distinguishing the relative importance of adsorptive and degradative phenomena as possible factors in the loss of activity during storage of neutral solutions in glass. However, the radiochromatographic evidence suggests that the former is of major importance because it is unlikely that degradation products will occupy the same position as histamine on the TLC plate.

Preparation and storage of histamine standards and extracts in polyethylene containers has allowed us to demonstrate that no interference from histidine is detectable in the modified automated method for determining histamine at concentrations of histidine that are slightly in excess of those normally present in fresh mackerel extracts. Fuller details of the method and of the influence of other potentially interfering components will be published later.

REFERENCES

(1) Gilbert, R. J., Hobbs, G., Murray, C. K., Cruickshank, J. G., & Young, S. E. J. (1980) *Br. Med. J.* **281,** 71–72

(2) Staruszkiewicz, W.F., Jr, Waldron, E. M., & Bond, J. F. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1125–1130

(3) Taylor, S. L., & Lieber, E. R. (1977) *J. Food Sci.* **42,** 1584–1586

(4) Staruszkiewicz, W. F., Jr (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1131–1136

(5) Lerke, P. A., & Bell, L. D. (1976) *J. Food Sci.* **41,**

1282–1284
(6) Taylor, S. L., Lieber, E. R., & Leatherwood, M. (1978) *J. Food Sci.* **43,** 247–250
(7) Evans, D. P., Lewis, J. A., & Thomson, D. S. (1973) *Life Sci.* **12,** Part II, 327–336
(8) von Redlich, D., & Glick, D. (1969) *Anal. Biochem.* **29,** 167–171
(9) Bosse, J.-A., & Wassermann, O. (1967) *Med. Pharmacol. Exp.* **16,** 459–461
(10) Hall, T. C., & Cocking, E. C. (1965) *Biochem. J.* **96,** 626–633
(11) Bush, E. T. (1968) *Int. J. Appl. Radiat. Isotopes* **19,** 447–452
(12) Hardy, R., & Murray, J. (1978) in *Liquid Scintillation Counting*, Vol. 5, M. A. Crook, & P. Johnson (Eds.), Heyden & Son, London, UK, pp. 195–202
(13) Wigfield, D. C., & Srinivasan, V. (1974) *Int. J. Appl. Radiat. Isotopes* **25,** 473–482
(14) Crosby, N. T., Hunt, D. C., Philp, L. A., & Patel, I. (1981) *Analyst* **106,** 135–145
(15) Chambers, T. L., & Staruszkiewicz, W. F., Jr (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1092–1097

PRESERVATIVES AND ARTIFICIAL SWEETENERS

Automated High Pressure Liquid Chromatographic System for Determination of Mannitol, Sorbitol, and Xylitol in Chewing Gums and Confections

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A rapid, precise, and reproducible automated high pressure liquid chromatographic method was developed to determine xylitol in the presence of mannitol and sorbitol in chewing gums and confectionery products. A mobile phase of water and methanol elutes the polyols simultaneously from a cation-exchange column without pretreatment or derivatization. Injections into the liquid chromatograph were made by an autosampler, and data reduction was performed with a programmable electronic integrator. Average recoveries for one level each of mannitol, sorbitol, and xylitol in 6 replicate samples prepared by the standard addition technique were 102.5, 100.5, and 100.7%, respectively.

The polyhydric alcohols, xylitol, mannitol, and sorbitol, are used in pharmaceuticals, chewing gums, and confectionery food products alone or in combination as sugar substitutes. Various ion-exchange chromatographic methods have been reported by Goulding (1) and Verhaar and Dirkx (2) to separate these polyols, but not quantitatively or simultaneously. Schwarzenbach (3) separated them by high pressure liquid chromatography (HPLC) after forming the nitrobenzoate derivatives.

Xylitol and sorbitol were not separated by the HPLC method previously described by Samarco (4). By modifying the resin (as previously described (4)) and the mobile phase with methanol, xylitol, mannitol, and sorbitol could be separated and quantitated rapidly and accurately without pretreatment or derivatization. The method was then automated to permit continuous, unattended operation.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Model ALC 204/401 equipped with Model 401 differential refractometer, Model 6000 solvent delivery system (Waters Associates, Inc., Milford, MA 01752), Model 420 autosampler equipped with 100 μL injection loop (Perkin-Elmer Corp., Norwalk, CT 06856), and Model SP4100 computing integrator (Spectra-Physics, 2905 Stender Way, Santa Clara, CA 95051). Typical instrument operating conditions: flow rate 2.0 mL/min (ca 800 psig); temperature 55°C; refractive index detector attenuation X16; autosampler cycle time 38 min; chart speed 0.5 cm/min. Under these conditions, mannitol, xylitol, and sorbitol elute in ca 18, 22, and 26 min, respectively.

(b) *HPLC column.*—Empty column assembly (316SS) No. 98146, 3/8 in. × 2 ft with 10 μm end fittings (Water Associates).

(c) *Column packing.*—No. 147-2202 Aminex Q-15S, converted to calcium form (Bio-Rad Laboratories, Richmond, CA 84004).

(d) *Mobile phase.*—Water–methanol (65 + 35); filter individually through 0.45 μm pore membrane filters (Millipore Corp., Bedford, MA 01730).

(e) *Heat controller and heating tape.*—Thermolyne controller No. E-2023 and heating tape No. H2125 (Scientific Glass Apparatus, Bloomfield, NJ 07003).

(f) *Standard preparation.*—Prepare 3 standard mixtures of mannitol, xylitol, and sorbitol (ca 1, 2, and 3 mg/mL of mannitol; 5, 10, and 15 mg/mL of xylitol; and 40, 50, and 60 mg/mL of sorbitol) in water. Filter through 0.45 μm pore membrane for HPLC analysis.

Preparation of Resin and Column

Filter all reagents through 0.45 μm pore membranes. Wash ca 50 g Aminex Q-15S resin with three 1 L portions of water, decanting each time to remove fines. Add ca 1 L 1N HCl to the washed resin and heat to boiling on hot plate. Cool, filter through fine porosity sintered glass funnel under vacuum, and wash with two 200 mL portions of water. Add ca 1 L 1M $CaCl_2$, heat to boiling on hot plate, cool, filter, and wash with water as above. Wash with two 200 mL portions of water–methanol mobile phase. Slurry resin

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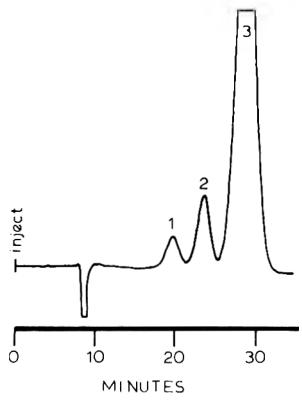

Figure 1. Liquid chromatographic separation of 1, 2 mg/mL of mannitol; 2, 10 mg/mL of xylitol; 3, 50 mg/mL of sorbitol in a mixed standard solution.

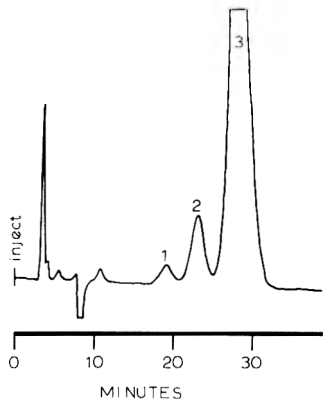

Figure 2. Liquid chromatographic separation of 1, 1.3 mg/mL of mannitol; 2, 6.8 mg/mL of xylitol; 3, 57.4 mg/mL of sorbitol in a sugarless chewing gum.

in 100 mL water–methanol mobile phase and slurry-pack column under pressure.

Sample Preparation

Accurately weigh about 2 g sugarless chewing gum or about 1 g sugarless candy into 50 mL centrifuge tube. Add 10.0 mL toluene and 20.0 mL water. Shake mechanically until gum is dispersed. Centrifuge, and filter portion of aqueous phase through 0.45 μm pore membrane for HPLC analysis.

Liquid Chromatography and Data Reduction

Once initiated, the following analysis is performed without operator intervention:

A 100 μL portion of each standard mixture is automatically injected into the liquid chromatograph. The peak area of each component is obtained by a computing electronic integrator. The integrator is preprogrammed to perform linear regression analysis of peak area vs mg/mL, and a calibration curve is obtained for each component.

In a similar manner, a sample preparation is injected. The amounts of xylitol, sorbitol, and mannitol are computed automatically by the integrator which interpolates each component from its respective calibration curve. A full report can be generated by entering sample weights and dilution factors.

Composite Sample Analysis

A batch of sugarless chewing gum having a previously analyzed composition of 1.3% mannitol, 6.8% xylitol, and 57.4% sorbitol was cut into small pieces ($\frac{1}{4} \times \frac{1}{4}$ in.) and mixed. Six replicate samples were prepared from the composite sample.

The recovery study was performed on the composite sample, by the standard addition technique. Six samples, representing one-half the determined concentration (1 g), were accurately weighed and ca 550 mg sorbitol, ca 75 mg xylitol, and 20 mg of mannitol were added to each sample which was then ready for HPLC analysis as above.

Results and Discussion

Typical separations of a mixed standard (Figure 1) and a chewing gum sample (Figure 2) show retention times of 18, 22, and 26 min for mannitol, xylitol, and sorbitol, respectively. A blank chewing gum showed no interferences under the peaks of the components of interest.

The averages for the analysis of 6 replicates of the composite chewing gum sample were 1.3 (mannitol), 6.8 (xylitol), and 57.4% (sorbitol). The relative standard deviations were 3.2 (mannitol), 4.4 (xylitol), and 0.9% (sorbitol).

The average recoveries for 6 replicate samples prepared by the standard addition method were 102.5 (mannitol), 100.7 (xylitol), and 100.5% (sorbitol). The relative standard deviations were 3.7 (mannitol), 2.6 (xylitol), and 1.4% (sorbitol).

A variety of commercially available chewing gums and confectionery products were analyzed by this method (Table 1). As a rule, in our laboratory, especially when dealing with samples of unknown concentrations, the approximate concentrations are determined first. Then, standard concentrations are prepared at 50, 100, and 150% of expected amount. Exact concentration is found by linear regression analysis.

Table 1. Analysis of some sugarless gum and confectionery products

Product	Mannitol, %	Xylitol, %	Sorbitol, %
Gum brand A			
Bubble gum flavor	2.3	10.0	55.5
Fruit flavor	2.1	9.4	53.6
Spearmint flavor	2.5	9.4	55.9
Gum brand B			
Bubble gum flavor	13.8	7.9	42.5
Fruit flavor	1.3	6.8	57.4
Spearmint flavor	0.8	7.1	57.0
Gum brand C	0.7	44.7	32.8
Gum brand D	—	71.9	8.2
Candy brand X	3.4	53.6	41.8

Ease of sample preparation, simultaneous determination of the 3 polyols, automated analysis, and data reduction afford a considerable time savings and permit a high throughput of samples per day.

It has been our experience that the column itself is very durable. After prolonged column usage, the resin may be removed from the column, regenerated, and repacked with no noticeable deleterious effects.

REFERENCES

(1) Goulding, R. W. (1975) *J. Chromatogr.* **103,** 229–239
(2) Verhaar, L. A. T., & Dirkx, J. M. H. (1977) *Carbohydr. Res.* **59,** 1–10
(3) Schwarzenbach, R. (1977) *J. Chromatogr.* **140,** 304–309
(4) Samarco, E. C. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1318–1320

MICROBIOLOGICAL METHODS

Recovery of Eggs of Two Parasitic Nematodes, *Ascaris* sp. and *Trichuris* sp.: Interlaboratory Study

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An interlaboratory study was conducted to determine the effectiveness of the Nacconol ether centrifugation method for recovering parasitic nematode eggs from 3 contaminated products: a crop (cabbage), a sludge fertilizer (Milorganite), and a sewage effluent (Minneapolis). Six replicate samples for each of the 3 products were seeded with eggs at 3 different levels: 200 *Ascaris suis* and 8 *Trichuris muris;* 15 *A. suis* and 15 *T. muris;* 8 *A. suis* and 180 *T. muris.* Recovery was low for all samples except sewage effluent, in which recoveries greater than 100% in 2 samples resulted from the misidentification of arthropod eggs as *Ascaris* sp. The average mean percent recovery for the other samples was 22.53. Repeatability for replicate samples and reproducibility of results by individual laboratories were poor, and the method is not recommended for quantitative estimates of nematode egg contamination of foods and food-contact materials. However, the Nacconol ether centrifugation method can be used as an all-or-none test. (Only 13% of 1146 counts were falsely negative.) Of 69 samples, only 4 were falsely negative for *A. suis* eggs and only 1 was falsely negative for *T. muris* eggs in counts of 6 replicates.

Eggs of the pathogenic nematode parasites *Ascaris* spp. and *Trichuris* spp. survive common methods of sewage treatment and can contaminate food crops fertilized with sewage sludge or irrigated with sewage effluent. Diverse methods have been tried for recovering parasitic nematode eggs from the feces of infected hosts or from soils, waters, and vegetation where feces may be deposited. Some of these techniques were quantitative; others were used in an all-or-none manner. To select one technique for an interlaboratory study, 4 of the more common methods were compared in previous studies: zinc sulfate flotation (1), saline centrifugation (1), Nacconol ether centrifugation (2), and the water trough method (3). Because Nacconol ether centrifugation gave the best results (e.g., recoveries from cabbage exceeded 90%), it was the method selected for use in this interlaboratory study.

Sample Preparation and Shipment

Samples of Milorganite (a dried fertilizer product prepared from Milwaukee, WI, municipal sludge), liquid sewage effluent (from Minneapolis, MN), and shredded cabbage were seeded with 3 levels of *A. suis* and *T. muris* eggs. These eggs are considered to represent other species in the genera *Ascaris* and *Trichuris*, and hereafter will be referred to by the generic name alone. For even distribution, the *Ascaris* eggs had been treated with 0.2% sodium hypochlorite (2). Seeding levels were 200 *Ascaris* and 8 *Trichuris*, 15 *Ascaris* and 15 *Trichuris*, 8 *Ascaris* and 180 *Trichuris*/10 g sample. Seeded samples were checked by Minneapolis Center for Microbiological Investigations to establish that the eggs were evenly distributed. Six replicate samples at each seeding level were weighed into polyethylene bottles, placed into shipping containers (refrigerated at 0–6°C with gel packs), and shipped to each laboratory by air express. On arrival, the temperature was recorded and the sample was stored in a refrigerator until analysis. Participants were asked to begin analysis as soon as possible after receiving the sample.

Method

Reagents and Apparatus

(a) *Formalin solution.*—10%. Add 730 mL water to 270 mL of a 37% stock formaldehyde solution.

(b) *Nacconol 35 SL.*—Sodium linear alkylate sulfonate, 35% active (Stepan Chemical Co., Northfield, IL).

(c) *Nacconol solution.*—0.008%. 3.7 mL Nacconol 35 SL in 16 L physiological saline.

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(d) *Gridded tissue culture dishes.*—Falcon Integrid 3030 (Becton, Dickinson & Co., Oxnard, CA).

(e) *Bolting cloth.*—100 μm pores. Nitex HC3-100 (Nitex Corp., Bern, Switzerland).

(f) *Pasteur pipets.*—229 mm, with cotton plug and rubber bulb.

Determination

Add 85 mL 0.008% Nacconol solution to subsample in plastic bottle. Cap and shake 25 times in 30 cm arc, 25 shakes in about 7 s. Pour contents of bottle through soaked piece of bolting cloth into 450 mL beaker. Soak bolting cloth in 0.008% Nacconol immediately before use. Rinse bottle with two 50 mL portions of 0.008% Nacconol. Pour rinses through bolting cloth into beaker. Stir contents of beaker with glass stirring rod and pour into 15 mL conical centrifuge tubes. Centrifuge tubes 4 min at 2000 rpm and discard supernatant. With Pasteur pipet, transfer sediment into 4 of the 15 mL conical centrifuge tubes. Fill the 4 tubes with 0.008% Nacconol and centrifuge at 2000 rpm. Discard supernatant and add 3 mL 10% formalin to each tube. Resuspend sediment on Vortex mixer and loosen from bottom of tube with 4 mm glass rod. Add another 5 mL 10% formalin to each tube and let stand 4 min. Under chemical hood, add 3 mL ether to each tube. Stopper tubes with size 0 rubber stopper and shake vigorously 30 s. Centrifuge all tubes 4 min at 2000 rpm. Under the hood, pour supernatant into safety can; add 3 mL 0.008% Nacconol to each tube and resuspend sediment. For liquid sludge, combine resuspended contents of 2 tubes into Petri dish for total of 2 Petri dishes/subsample, or combine all 4 tubes into 1 Petri dish if amount of background material is small. For cabbage, combine resuspended contents from all 4 tubes into 1 Petri dish. For Milorganite, pour resuspended contents of each tube into separate Petri dishes. If background material is too heavy, dilute contents into more Petri dishes. To count the recovered parasites, scan entire Petri dish with inverted microscope and record results.

Results and Discussion

Table 1 lists mean percent recovery (MPR) for a given nematode egg type at a specified seeding level, the standard deviation (SD) and coefficient of variation (CV) that reflect the repeatability or variation among replicate samples for a given egg type and seeding level, and the SD and CV that reflect the reproducibility of the results among laboratories and replicate variations.

The average percent recovery of *Ascaris* and *Trichuris* eggs at each of the seeding levels (low, mid, high) were as follows: For Milorganite samples at the high seeding level, MPR for *Trichuris* was significantly greater than for *Ascaris* ($P < 0.01$). There were no significant differences between the MPRs for *Trichuris* and *Ascaris* at the low and mid levels ($P < 0.25$). For liquid sludge, the MPR for *Ascaris* was significantly higher than that for *Trichuris* at the low and mid levels ($P < 0.05$). There was not a significant difference between the MPRs for *Ascaris* and *Trichuris* at the high level ($P > 0.10$). For shredded cabbage samples, the MPRs for *Trichuris* were significantly higher than those for *Ascaris* at the high and mid levels ($P < 0.01$). There was no significant difference between the MPRs for *Ascaris* and *Trichuris* at the low level ($P > 0.01$). For Milorganite samples, MPRs were significantly less than 100% for both egg types at all levels ($P < 0.01$). For liquid sludge, the MPR for *Ascaris* was significantly less than 100% at the high level, greater than 100% at the medium and low levels ($P < 0.05$); the MPR for *Trichuris* was significantly less than 100% for all levels ($P < 0.01$). For cabbage samples, the MPRs for *Ascaris* and *Trichuris* were significantly less than 100% for all levels ($P < 0.01$).

Interpretation of Results

The Nacconol ether centrifugation method can be used as an all-or-none test. Of 1146 counts, only 149, or 13%, were negative. When 6 replicates were counted for each product at each seeding level, the number of false negatives was 4 for the *Ascaris* and 1 for the *Trichuris* per 69 different samples. If only 3 replicates were done on each sample, there would probably be 19 false *Ascaris* negatives and 6 false *Trichuris* negatives per 69 samples.

The low MPRs (except for *Ascaris* in liquid sludge) may be due to the difficult-to-overcome adherence of eggs to all types of surfaces and particles. This appears to be the most likely explanation for the great variance of the results, especially for the "sticky" *Ascaris* eggs. Apparently, the attempt to remove the *Ascaris* egg's adhesive coat with sodium hypochlorite before seeding succeeded only in part. Manual shaking of the sample 25 times in a 1 ft arc also may have contributed to the low MPR. The greater-than-seeded numbers of *Ascaris* reported in liquid sludge were probably due to misidentification of other bodies (mite eggs) in these samples as *Ascaris* eggs.

Table 1. Interlaboratory results for recovery of *Ascaris* sp. and *Trichuris* sp. eggs from 3 sample types

	Seeding level[a]					
	Ascaris eggs			*Trichuris* eggs		
	200	15	8	8	15	180
Parameter	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
			Milorganite			
Mean % recovery	6.24	30.28	27.43	20.66	21.67	37.90
SD						
Repeatability	3.66	14.54	26.17	15.93	18.76	7.26
Reproducibility	6.07	23.64	38.66	21.99	35.14	22.28
CV						
Repeatability	58.65	48.02	95.41	77.11	86.57	19.16
Reproducibility	97.28	78.03	140.94	106.44	162.16	58.78
			Liquid Sludge			
Mean % recovery	17.92	132.40	304.55	25.38	25.15	23.75
SD						
Repeatability	4.43	65.90	158.34	19.68	19.77	6.09
Reproducibility	20.74	164.89	393.03	32.09	22.54	13.48
CV						
Repeatability	24.72	49.77	51.99	77.54	78.61	25.64
Reproducibility	115.74	124.54	129.05	126.44	89.62	56.76
			Cabbage			
Mean % recovery	6.83	13.06	18.66	24.65	31.11	29.86
SD						
Repeatability	6.70	13.91	19.14	18.09	16.81	14.34
Reproducibility	10.27	24.28	32.07	19.56	21.24	18.12
CV						
Repeatability	98.09	106.54	102.57	73.38	54.05	48.02
Reproducibility	150.22	185.94	171.83	79.35	68.28	60.68

[a] Samples 1, 2, and 3 were seeded with both *Ascaris* and *Trichuris* eggs at the levels shown.

Recommendation

Nacconol ether centrifugation is recommended as a qualitative test for determining the presence of *Ascaris* sp. and *Trichuris* sp. eggs in liquid or on solid food-contact materials (crop irrigants and fertilizers) and on foods. However, for better quantitative recovery an improved method is needed. This method should then be compared collaboratively with the Nacconol ether centrifugation method, using *Ascaris* and *Trichuris* eggs seeded at the mid-level (15 eggs) onto the 3 products (Milorganite, liquid sludge, shredded cabbage). Steps for implementing the recommendation have been undertaken.

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References

(1) Faust, E. C., Russell, P. F., & Jung, R. C. (1970) *Craig and Faust's Clinical Parasitology*, 8th Ed., Lea & Febiger, Philadelphia, PA, 870 pp.

(2) Jackson, G. J., Bier, J. W., & Rude, R. A. (1978) in *Proceedings of the Conference on Risk Assessment and Health Effects of Land Application of Municipal Waste Water and Sludges*, B. P. Sagik & C. A. Sorber (Eds.), Center for Applied Research and Technology, University of Texas at San Antonio, San Antonio, TX, pp. 116–127

(3) Beer, R. J. S. (1972) *Parasitology* **65**, 343–350

COSMETICS

Fluorometric Determination of Dehydroacetic Acid in Mascara Products

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A fluorometric method for the determination of dehydroacetic acid in mascara products was developed. The method is based on the formation of the fluorophore γ-pyroxonium borate, which occurs when dehydroacetic acid is reacted with sulfuric and boric acids. Fluorescence intensity is measured and the amount of dehydroacetic acid is determined from a standard calibration curve. Studies were conducted at the 0.1, 0.05, and 0.01% levels. Recoveries ranged from 88 to 106% with an overall average of 99%.

Dehydroacetic acid (DHA) (methylacetopyrone), along with one or more other chemical preservatives, is used in mascara formulations as a preservative. Effective preservative systems are essential in such products to prevent the growth of harmful microorganisms, which can be introduced into a product during manufacture or repeated customary use. Serious eye injuries have resulted when mascara products contaminated with *Pseudomonas aeruginosa* have come into contact with the scratched or damaged cornea of the eye (1). Our laboratory is frequently requested to identify preservatives and determine their concentrations in products which have been found to be contaminated with microorganisms. This information is needed to determine if the contaminated product under investigation actually contains the preservatives specified in the formulation.

Nagasawa et al. (2) and Wilson (3) reported methods for the thin layer chromatographic (TLC) identification of DHA. Ultraviolet (UV) spectrometry (4, 5) and gas chromatography (6) have been used to determine DHA. These methods, when applied to mascara products, require extensive sample preparation to eliminate interfering compounds. After some investigation, it was determined that a more selective method requiring minimal sample preparation was needed.

When treated with sulfuric acid, DHA undergoes isomerization and decarboxylation to form 2,6-dimethyl-γ-pyrone (7). The γ-pyrone forms fluorescent, stable oxonium salts with mineral acids. Shibazaki (8, 9) isolated DHA from foods by steam distillation and determined it fluorometrically as the γ-pyrone borate salt.

The method that we developed involves dispersing a sample of mascara in warm methanol and diluting to a known volume with methanol. After the pigment has settled, an aliquot of the supernatant liquid is diluted with water to a known volume. An aliquot of this solution is reacted with sulfuric and boric acids according to the general procedure of Shibazaki to form the fluorescent oxonium salt. Fluorescence intensity is then measured, and the amount of DHA present is determined from a standard emission curve. The accuracy of the method was verified by conducting recovery studies at 3 concentration levels.

METHOD

Apparatus

(a) *Fluorescence spectrophotometer.*—Model MPF-3 with Model 150 power supply (Perkin-Elmer Corp., Norwalk, CT 06856), or equivalent. Operating conditions: excitation, 325 nm; emission, scan from 330 to 500 nm; excitation and emission slits, 10 nm; filter, 350 nm. Let instrument warm up for 30 min.

(b) *Constant temperature bath.*—With accuracy of ±1°C.

Reagents

(a) *Acetic acid, boric acid, methanol, and sulfuric acid.*—ACS reagent grade, or equivalent.

(b) *Boric acid solution.*—Dissolve 1 g boric acid in 100 mL water and mix.

(c) *Dehydroacetic acid (DHA) standard solutions.*—Prepare fresh stock solutions weekly. (1) *Stock solution A.*—Accurately weigh ca 20 mg DHA (Eastman Kodak Co., Eastman Organic Chemicals, Rochester, NY 14650, Cat. No. 1624). Quantitatively transfer to 100 mL volumetric flask, dilute to volume with water, and mix. (2) *Stock solution B.*—Pipet 1.0 mL stock solution

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A into 100 mL volumetric flask, dilute to volume with water, and mix.

Preparation of Standard Calibration Curve

Pipet 1.0, 2.0, 3.0, 4.0, and 5.0 mL aliquots of stock solution B into separate 100 mL volumetric flasks, dilute to volume with water, and mix.

Pipet 1.0 mL of each of above solutions into separate 10 mL volumetric flasks. Place volumetric flasks into ice-water slurry and slowly add 7 mL concentrated sulfuric acid, using Pasteur pipet or buret. Agitate flasks to aid cooling. Add 1.0 mL boric acid solution to each and mix well. Cool volumetric flasks to 20°C, dilute to volume with concentrated sulfuric acid, and mix well. Incubate standards at 35°C for 20 min in water bath. Cool to room temperature (ca 2 h), and then determine fluorescent emission. Adjust sensitivity of fluorescence spectrophotometer so that the most concentrated standard is on scale. Prepare standard curve by plotting micrograms of DHA vs peak height of emission band. A linear relationship should be obtained.

Preparation of Sample

Accurately weigh 1 g mascara into 50 mL beaker, and add 1 mL acetic acid and 15 mL methanol. Warm mixture on steam bath with stirring until sample is dispersed. With the aid of several small portions of methanol, quantitatively transfer mixture to 100 mL volumetric flask, dilute to volume with methanol, and mix thoroughly. Let pigment settle, and then pipet 1.0 mL of the clear supernatant liquid into 100 mL volumetric flask. Dilute to volume with water and mix. Pipet 1.0 mL aliquots of this solution into each of three 10 mL volumetric flasks, and prepare the fluorescent pyrone derivative, using procedure given under Preparation of Standard Calibration Curve.

Determination

Determine fluorescent emission intensity of sample solution, using operating conditions used to prepare standard curve. If emission values do not fall within or near range of standards, make appropriate dilutions of final sample solution and prepare fluorescent derivative again. Average sample emission values and determine amount of DHA from standard curve. Calculate percent DHA in sample according to following equation:

$$\% \text{ DHA} = W_d \times D / W_s \times 10^4$$

where W_d is the weight of DHA from standard curve (μg), D is the dilution factor, and W_s is the weight of the sample (g).

Table 1. Recoveries of DHA added to mascara

Sample[a]	Added, mg	Found, mg	Rec., %
1	1.00	0.96	96
2	1.00	0.98	98
3	1.00	1.00	100
4	0.50	0.52	104
5	0.50	0.53	106
6	0.50	0.52	104
7	0.10	0.099	99
8	0.10	0.092	92
9	0.10	0.088	88
Mean			98.6
SD (σ)			5.9

[a] 1 g samples of mascara; one determination per sample.

Results and Discussion

The proposed method was evaluated by conducting recovery studies with a laboratory-prepared mascara to which known amounts of DHA had been added. The mascara, typical in composition to commercial formulations, was composed of water, triethanolamine, glyceryl monostearate, stearic acid, cetyl and stearyl alcohols, acrylic-acrylate copolymer, and pigment (Fe_3O_4). DHA was added to 1 g samples of mascara in amounts corresponding to 0.1, 0.05, and 0.01%. Results of the study are shown in Table 1.

Initial investigations involved the determination of DHA by UV spectrometry after sample preparation by column partition chromatography. However, recoveries of added DHA were low and erratic. Column eluates frequently contained interfering components that absorbed in the UV region of interest. These studies indicated that a more selective method for determining DHA was required.

Preliminary work involving the fluorescent method was directed toward determining the linearity and reproducibility obtainable with standard solutions of DHA. Results indicated that the DHA conversion to the fluorescent derivative was quantitative or at least reproducible and linear over the range studied.

As a result of Raman scatter, the standard emission curve does not intersect the origin. This is expected because of the close proximity of the excitation and emission bands, 325 and 368 nm, respectively. The stability of the fluorophore was determined by periodic measurement of emission intensity over a 48 h period. There was no significant change in emission values.

Several sample preparation methods were evaluated. When mascara was dispersed in methanol and filtered through a fine glass frit by suction, much of the pigment passed through the filter. The retained pigment soon clogged the frit and prevented filtration. Experiments conducted with various filters of known pore sizes indicated that much of the pigment consisted of particles less than 1 μm in diameter, thus making the extraction and filtration approach impractical. When DHA was separated from the mascara by steam distillation from aqueous suspensions saturated with sodium chloride, clean distillates containing no interfering components were obtained. However, maximum recoveries were only 75%. The sample preparation procedure of the developed method is simple and avoids the problems associated with filtration. Pigments usually settled in a few hours. In some commercial mascara samples, a small amount of pigment remained in suspension after 4 to 5 h; however, after serial dilution and preparation of the fluorescent derivative, no effects on recoveries of added DHA were noted.

In summary, a simple, selective method for the fluorometric determination of DHA in mascaras has been developed. The accuracy of the method was verified by conducting recovery studies at 3 levels that cover the normal concentration range of DHA in these products. Results indicate that the method is adequate for the determination of DHA in mascaras. With minor modifications in sample preparation technique, this method can be applied to other products.

REFERENCES

(1) *Fed. Regist.* (October 11, 1977) **43,** 54837–54838
(2) Nagasawa, K., Yoshidome, H., & Takeshita, R. (1969) *J. Chromatogr.* **43,** 473–479
(3) Wilson, C. H. (1975) *J. Soc. Cosmet. Chem.* **26,** 75–81
(4) Sayato, Y., Nakata, M., Okazaki, M., & Sayo, T. (1966) *Shokuhin Eiseigaku Zasshi* 7(3), 199–203; *Chem. Abstr.* (1967) **67,** 98972z
(5) Sheppard, E. P., & Wilson, C. H. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 937–940
(6) Wariishi, K., & Bando, H. (1975) *Tokushima Bunri Daigaku Kenkyu Kiyo* **12,** 29–34; *Chem. Abstr.* (1978) **89,** 22371m
(7) Fieser, L. F., & Fieser, M. (1956) *Organic Chemistry,* 3rd Ed., Reinhold Publishing Corp., New York, NY, p. 816
(8) Shibazaki, T. (1968) *Yakugaku Zasshi* **88**(11) 1398–1403; *Chem. Abstr.* (1969) **70,** 66853g
(9) Shibazaki, T. (1975) *Iyakuhin Seiza No Bunseki* 110–124; *Chem. Abstr.* (1976) **84,** R126301q

VITAMINS AND OTHER NUTRIENTS

Radioisotope Dilution Technique for Determination of Vitamin B_{12} in Foods

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A radioisotope dilution (RID) method for the determination of vitamin B_{12} is presented. The method combines a standard extraction procedure (AOAC 43.108, 12th ed.) with a commercially available RID assay kit. The method was evaluated on a wide range of fortified and unfortified food products. Recovery studies on both groups yielded average recoveries of 98.1 and 95.8%, respectively. Reproducibility data generated from replicate analyses on both groups gave a relative standard deviation of 6.9% for the fortified group and 9.2% for the unfortified group. For the samples studied, the mean vitamin B_{12} content determined by the RID method was 8.01 μg/100 g vs a mean of 7.54 μg/100 g by the AOAC microbiological method; the correlation coefficient was $r = 0.983$.

The determination of vitamin B_{12} is of considerable interest to the food industry. Many methods exist, and these have been appropriately reviewed (1). Because of the low levels of vitamin B_{12} in foods (2), microbiological procedures have long been the method of choice for the food industry. However, these procedures generally are time consuming, requiring from 2 to 4 days to complete, and can be quite technique-specific.

For many years, radioisotope dilution (RID) techniques have been used by clinical laboratories to monitor serum levels of vitamin B_{12}; RID assays are rapid, sensitive, and uninfluenced by antibiotics and other drugs (3). These techniques are based on the principles of saturation analysis described by Ekins (4), and a vitamin B_{12} radioassay described by Lau et al. (5). Measured amounts of serum, buffer, cyanide, and radioactive-labeled (^{57}Co) vitamin B_{12} are mixed and heated to dissociate the various forms of vitamin B_{12} from the binding proteins present and convert them to a single form of the vitamin, cyanocobalamin. This mixture is then incubated with a measured amount of a glycoprotein, intrinsic factor (IF), which serves as a nondiscriminative binding agent for both labeled and unlabeled cyanocobalamin. During incubation, labeled and unlabeled cyanocobalamin compete for IF on the basis of their concentrations. Following incubation, IF-bound cyanocobalamin and unbound cyanocobalamin are separated, and the level of radioactivity is measured in either fraction. The concentration of vitamin B_{12} in the serum can then be determined from a standard curve, by comparing the degree to which the binding of the labeled vitamin B_{12} is inhibited by the unlabeled vitamin B_{12} present in the serum. Recently, this technique has been used for determination of vitamin B_{12} in a variety of substances, including food extracts (6, 7) and natural waters (8).

The purpose of this study was to explore the application of a commercially available vitamin B_{12} RID assay kit, developed for clinical applications, to food products. We felt that the combination of this widely accepted technique with a standard extraction procedure (9) should give a method applicable to a broad range of food products, with accuracy and precision similar to that of the clinical techniques, while significantly reducing the time and cost of the assays.

METHOD

Apparatus

(a) *Gamma counter.*—Picker Compac 120 equipped with PALL-M data analyzer (Picker Corp., Northford, CT 06472), or equivalent.

(b) *Centrifuge.*—Beckman TJ-6 equipped with TH-4 rotor and No. 339285 Maxi-carriers (Beckman Instruments, Inc., Palo Alto, CA 94304), or equivalent.

(c) *Mixer.*—Vortex, Genie K-550-G (Scientific Industries, Inc., Bohemia, NY 11716), or equivalent.

(d) *Food processor.*—Moulinex 354 (Moulinex Products, Inc., Virginia Beach,VA 23454), or equivalent.

(e) *Heating bath.*—Thelco 182 (GCA/Precision Scientific, Chicago, IL 60647), or equivalent.

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(**f**) *Reaction tubes.*—12 × 75 mm polypropylene (Bio-Rad Laboratories, Richmond, CA 94804), or equivalent.

Reagents

(**a**) *Extraction solvent.*—Dissolve 13 g anhydrous sodium phosphate dibasic, 12 g citric acid (monohydrate), and 10 g sodium metabisulfite in water. Dilute to 1 L with water. Prepare fresh for each run.

(**b**) *Phosphate buffer.*—pH 7.0. Dissolve 9.1 g anhydrous potassium phosphate monobasic and 18.9 g anhydrous sodium phosphate dibasic in water. Dilute to 1 L with water.

(c) *Ethanol.*—25%. Add 250 mL absolute ethanol to 1 L flask. Dilute to volume with water.

(**d**) *Radioassay reagents.*—Contained in the Quanta-Count II™ test kit (Bio-Rad Laboratories). (*1*) *Vitamin B_{12} binding protein stock solution.*—Reconstitute with 10 mL water. Agitate gently to dissolve. Refrigerated solution is stable 30 days. (*2*) *Vitamin B_{12} binding protein working solution.*—Dilute 1 part binding protein stock solution with 4 parts water. Prepare fresh daily. (*3*) *Dithiothreitol (DTT)-borate buffer solution.* Reconstitute one vial of DTT (10 μg DTT/mL KCN) with 4 mL borate buffer drawn from 120 mL borate buffer supply. Agitate gently to dissolve. Quantitatively add all of dissolved DTT to remaining borate buffer, and mix well. Refrigerated solution is stable 30 days. (*4*) *Working tracer solution.*—10 μL vitamin B_{12} (^{57}Co) solution/mL DTT-borate buffer solution. Prepare immediately before required addition in experimental section: "Add 1 mL working tracer solution to each tube." (*5*) *Adsorbent tablets.*—Store at 10–25°C. Use as supplied.

(**e**) *Cyanocobalamin stock standard solutions.*—Use low actinic flasks. (*1*) *100 μg/mL.*—Accurately weigh 50 mg cyanocobalamin (ICN Pharmaceutical, Inc., Irvine, CA 92715; store in desiccator) into 500 mL volumetric flask. Dissolve and dilute to volume with 25% ethanol. Store in refrigerator. Solution is stable 6 months. (*2*) *10 μg/mL.*—Pipet 50 mL 100 μg/mL solution into 500 mL volumetric flask and dilute to volume with 25% ethanol. Store in refrigerator. Solution is stable 6 months.

(**f**) *Cyanocobalamin working standard solutions.*—Use low actinic flasks. (*1*) *1 μg/mL.*—Pipet 10 mL 10 μg/mL solution into 100 mL volumetric flask and dilute to volume with extraction solvent. Prepare fresh daily. (*2*) *10 ng/mL.*—Pipet 1 mL 1 μg/mL solution into 100 mL volumetric flask and dilute to volume with extraction solvent. (*3*) *Working solutions.*—Pipet 0, 1, 3, 5, 10, and 20 mL portions of 10 ng/mL solution into separate 100 mL volumetric flasks, and add 50, 49, 47, 45, 40, and 30 mL, respectively, of extraction solvent to prepare working solutions containing 0, 100, 300, 500, 1000, and 2000 pg/mL, respectively.

Preparation of Samples

(**a**) *Low moisture samples.*—Grind to pass 40 mesh sieve. Accurately weigh portion of sample (maximum weight 5 g) containing ca 70 ng B_{12} into 100 mL low actinic volumetric flask and add 50 mL extraction solvent. Agitate to disperse. Wash down sides of flask with minimum of distilled water.

(**b**) *High moisture samples (meats and prepared foods).*—Grind sample in food processor. Weigh portion of this slurry (maximum weight 5 g) containing ca 70 ng B_{12} into 100 mL low actinic volumetric flask and add 50 mL extraction solvent. Agitate to disperse. Wash down sides of flask with minimum of distilled water.

Experimental

Autoclave samples and working standard solutions 10 min at 121–123°C. Cool rapidly to room temperature and dilute to volume with phosphate buffer. Invert flasks 10 times to mix well, and filter solutions through Whatman No. 2 paper. Pipet 100 μL portion of filtrate for each sample or working standard solution into each of 2 reaction tubes. Add 1 mL working tracer solution to each tube. Vortex-mix tubes gently and place in 100°C heating bath for 15 min. Cool tubes rapidly to room temperature. Add 1 mL vitamin B_{12} binding protein working solution to each tube. Vortex-mix tubes well and incubate 45 min at room temperature. Add 1 adsorbent tablet to each tube and let tubes stand for 5 min. Vortex-mix 10 s, and let stand 5 min. Centrifuge tubes 10 min at 1500 RCF (relative centrifugal field in g). Discard liquid fraction by gently inverting tubes and touching each to adsorbent paper to remove last droplets.

Count ^{57}Co decay in precipitate for 1 min. If sample is outside standard curve range, dilute with zero standard and repeat procedure, beginning "Invert flasks 10 times . . .".

Determine vitamin B_{12} concentration in sample extracts by PALL-M data analyzer using 4-parameter logistic equation:

$$B = [a/(1 + bp^c)] + d$$

where B = counts per minute, p = concentration, pg/mL, and a, b, c, and d are adjustable parame-

ters determined by iterative methods. This equation has the advantage of eliminating the severe nonuniformity of variance introduced by the logit transformation (10). Alternatively, concentration can be determined by any of several methods reviewed elsewhere (11).

Calculate vitamin B_{12} level in products as follows:

$$\text{vitamin } B_{12}, \mu g/100\ g = C/(100 \times W)$$

where C = concentration, pg/mL; W = sample weight, g; and 100 is a combined factor that takes into account 100 mL extract, level reported per 100 g, and conversion of pg to μg.

Results and Discussion

Preliminary experiments with 0.1N HCl as the extraction medium gave erratic results, while the AOAC extraction procedure was reproducible and worked well on all food samples analyzed. The use of 50 mL extraction solution resulted in the quantitative extraction of cobalamins from as much as 5 g sample. Occasionally, gelling of the diluted extract made filtration difficult. This was easily remedied by centrifuging the extract at 1500 × g for 10 min before filtration.

The problems with incomplete extraction noted in a previous paper (7) were not encountered here. This is probably due to the use of the more rigorous autoclave extraction, rather than the heating bath method.

The use of a benzyl alcohol partitioning step to remove interfering compounds, previously reported (6, 8), did not appear necessary in this study. We speculate that the rigorous extraction coupled with the increased specificity of the highly purified intrinsic factor used effectively eliminates interference by other biochemical compounds.

Twenty different fortified and unfortified food products were analyzed by the RID method and by the AOAC microbiological method (12). Duplicate and triplicate analyses of each sample were conducted by the RID method on different days to determine the reproducibility of the method. Recovery data were obtained by spiking each of the samples with cyanocobalamin at levels approximately equal to their original B_{12} content. The samples were then processed through the entire analytical sequence.

Reproducibility results for the 8 fortified foods analyzed indicated a relative standard deviation of 6.9% (10 degrees of freedom). Recoveries were essentially quantitative (98.1 ± 2.9%) for the 8 samples. Results are shown in Table 1.

Results for the 12 unfortified products indicated a relative standard deviation of 9.2% (17 degrees of freedom). Again excellent recoveries were observed with an average of 95.8% (SD 6.5%). Results are shown in Table 2.

The combined results from both the fortified and unfortified samples were compared with results obtained by analyzing each of the samples by the AOAC microbiological method. The data from Tables 1 and 2 illustrate that the RID method compares quite favorably with the AOAC method, exhibiting a correlation coefficient of 0.983 with a linear regression equation of $y = 0.94x + 0.04$. The mean vitamin B_{12} content determined by the RID method was 8.01 $\mu g/100$ g compared with the mean determination of 7.54 $\mu g/100$ g by the AOAC microbiological method.

The overall procedure described gives good results for a wide variety of products. Reproducible results for B_{12} in sample extracts could be obtained at levels as low as 50 pg/mL, corresponding to 0.10 μg $B_{12}/100$ g (5 g sample

Table 1. Comparison of RID with microbiological method for vitamin B_{12} determination in fortified food products

Sample	No. of samples	RID B_{12}, $\mu g/100$ g (av. ± SD)	RID Av. rec., %	Micro., av. B_{12} ($\mu g/100$ g)
Drink mix	3	23.5 ± 1.21	96.7	22.5
Fortified bar	2	10.6 ± 0.21	95.5	13.5
Dog food	2	7.45 ± 0.10	93.9	9.57
Wheat cereal	3	28.0 ± 1.01	103.3	28.4
Corn cereal	2	8.38 ± 0.86	98.1	7.12
Oat cereal	2	10.4 ± 0.14	99.2	8.96
Meal composite	2	4.38 ± 0.11	98.1	4.37
Imitation bacon	2	11.7 ± 1.56	100.0	8.60
Overall mean rec. ± SD			98.1 ± 2.9	
RSD (10 degrees of freedom)			6.9%	

Table 2. Comparison of RID with microbiological method for vitamin B_{12} determination in unfortified food products

Sample	No. of samples	RID		Micro.,
		B_{12}, μg/100 g (av. ± SD)	Av. rec., %	av. B_{12}(μg/100 g)
Casein	3	1.24 ± 0.17	83.0	1.18
Cheddar cheese	3	0.665 ± 0.048	95.7	0.73
Powdered egg yolk	2	6.15 ± 0.31	89.3	5.37
Nonfat dry milk	3	2.23 ± 0.14	101.2	2.11
Clams	2	14.6 ± 1.13	103.8	10.2
Pollock	2	1.61 ± 0.23	99.3	1.85
Whiting	2	0.626 ± 0.013	95.3	0.66
Hamburger	2	1.48 ± 0.21	106.3	1.40
Pork chop	2	0.559 ± 0.001	91.5	0.52
Chicken liver	3	25.7 ± 0.87	94.0	22.9
Chicken	2	0.347 ± 0.076	91.4	0.29
Beef enchilada	3	0.55 ± 0.017	98.4	0.59
Overall mean rec. ± SD			95.8 ± 6.5	
RSD (17 degrees of freedom)			9.2%	

weight). This compares well with the generally quoted detection limit of 0.12 μg/100 g for the AOAC method (10). The minimal sample handling and workup required, as well as the rapid turnaround, make the RID method attractive for routine work.

REFERENCES

(1) Blake, C. J. (1977) *Brit. Food Manuf. Ind. Res. Assoc. Sci. Tech. Surveys* **95**, 16–21
(2) Marks, J. (1975) *A Guide to the Vitamins*, University Park Press, Baltimore, MD, pp. 117–124
(3) Grasbeck, R., & Salonen, E.-M. (1976) *Prog. Food Nutr. Sci.* **2** (5), 193–231
(4) Ekins, R. P. (1960) *Clin. Chim. Acta* **5**, 453–459
(5) Lau, K.-S., Gottlieb, C., Wasserman, L. R., & Herbert, V. (1965) *Blood* **26**, 202–214
(6) Beck, R. A. (1979) *J. Food Sci.* **44**, 1077–1079
(7) Richardson, R. J., Favell, D. J., & Jones, G. H. (1978) *Analyst* **103**, 865–868
(8) Beck, R. A. (1978) *Anal. Chem.* **50**, 200–202
(9) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Arlington, VA, sec. **43.108**
(10) Compac 120 Operators Manual (1979) Picker Corp., Northford, CT, pp. 33–34
(11) Skelly, D. S., Brown, L. P., & Besch, P. K. (1973) *Clin. Chem.* **19** (2), 146–186
(12) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Arlington, VA, secs. **43.105–43.109**

REFERENCE STANDARDS

Accuracy of Pesticide Reference Standard Solutions. Part I. Factors Affecting Organic Solvent Evaporation

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A gravimetric experiment was undertaken to identify the factors affecting solvent evaporation from analytical reference standard solutions and to establish the magnitude of the resultant solvent evaporation. The evaporation of organic solvent from standard solutions is affected by: (1) solvent, (2) container type, (3) solution volume, and (4) storage temperature. Solvents with low vapor pressure-to-surface tension ratios have low evaporation rates. Screw-cap containers with precision-made caps and seals have the lowest evaporation rates. The use of large-volume standard solutions reduces the percent solvent loss. Storage at refrigerator or freezer temperatures can reduce evaporation rate by as much as a factor of 7.

The integrity of reference standard solutions should be of primary importance to the analytical chemist. The accuracy of analytical determinations cannot be any better than the accuracy of the reference standard solution used for the analysis.

This laboratory serves as a quality assurance (QA) coordinating center for approximately 30 pesticide residue laboratories. We have often noticed incorrect results from audit sample exercises that could not be traced to laboratory handling error or quantitation technique. For example, results from the analyses of unknown standard solution QA samples are quite often lower than the formulation values. These low results are likely caused by the use of analytical reference standard solutions which have evaporated and therefore concentrated during storage.

A two-part investigation was therefore initiated to gain a better understanding of the various factors affecting the rate of solvent evaporation from the standard solution. This study was intended to identify the magnitude of solvent evaporation under normal laboratory conditions. The experiments were not intended to be a rigorous study of physical chemical properties. The factors studied include choice of solvent, type of container and closure, solution volume, and storage temperature. Part II (1) reports a long-term study of the chemical stability of solutions representing 4 classes of pesticide compounds.

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Experimental

Reagents and Equipment

(a) *Solvents.*—Acetone, benzene, ethyl ether (containing 2% ethanol), hexane, isooctane (2,2,4-trimethylpentane), methanol, methylene chloride, and toluene, distilled in glass (Burdick & Jackson Laboratories, Inc.).

(b) *Balance.*—Mettler Model PN163, with plastic dome to reduce air currents.

(c) *Containers.*—(*1*) Volumetric flasks with glass stoppers, clear glass, 100, 50, 10, and 5 mL.

(*2*) *Prescription bottles.*—Clear glass with cardboard-lined plastic caps, 3, 1, and ½ oz (Brockway Glass Co., Inc., Brockway, PA), and added Teflon cap liners (A. H. Thomas; No. 2390H).

(*3*) *Multivials.*—10 mL, clear glass (Supelco, Inc., Bellefonte, PA; No. 3-4579).

(*4*) *Serum bottles.*—10 mL, with Teflon-lined septa and seals (Wheaton Scientific, Millville, NJ; bottle No. 223739, septum No. 224167, seal No. 224182).

(*5*) *Small conical vials.*—Clear glass with Teflon-lined caps, or Mininert valves (Supelco, Inc.; 0.3 mL vials No. 3-3291, 0.1 mL vials No. 3-3293, and valves No. 3-3301).

(*6*) *Small flat-bottom vials.*—Clear glass with Teflon-lined caps, 1.5 and 4 mL (Wheaton Scientific; No. 225170 and 224882).

Experiment Design

The following evaporation rate experiments were performed in duplicate for 10 weeks each:

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(1) hexane and isooctane from an open 10 mL volumetric flask at ambient temperature (without replication, for one day only); (2) 8 solvents from 10 mL glass-stopper volumetric flasks at ambient temperature; (3) hexane from 4 sizes of glass-stopper volumetric flasks (5, 10, 50, and 100 mL) at ambient temperature; (4) hexane from 10 mL glass-stopper volumetric flasks at 3 storage temperatures (ambient, +4°C, and −15°C); (5) hexane and/or isooctane from several types of containers at ambient temperature.

At the beginning of each experiment, the appropriate glassware was weighed empty, filled with test solvent, and weighed again. Volumetric glassware was filled to the mark. Non-volumetric glassware, such as prescription bottles, was filled to give a head space equivalent to approximately 10% of the container volume. Samples were weighed weekly and returned to storage. Storage temperatures were recorded weekly.

The balance was carefully calibrated against the same 4 reference weights (chosen to be close to the weight of the full containers) before each data collection. To prevent containers from accumulating fingerprint oils, all containers were handled with nylon "lintless" gloves.

Before final data reduction, all data points were corrected for weekly variation by adding the calibration factor from the weighing of the reference weight closest to the weight of the container with solvent. Then all 11 data points (the weight at day zero and each of the 10 weekly weighings) were subtracted from the day zero weight and analyzed by least squares regression of the linear relationship and the linear transforms of the power, exponential, and hyperbolic functions.

The densities 0.664 and 0.689 g/mL were determined for hexane and isooctane, respectively, at ambient laboratory temperature at the beginning of the experiment

Results and Discussion

In all cases, the data analyzed best fit the linear relationship by least squares regression, indicating no change in the rate of solvent evaporation over the 10-week duration of the experiments.

Experiment 1. Evaporation Rates of Solvents from Open Volumetric Flasks

The evaporation rates of hexane and isooctane from an open 10.0 mL volumetric flask at ambient laboratory temperature (24°C) were established without experimental duplication to be 4.7 mL/wk and 1.8 mL/wk, respectively. The evaporation rate of hexane was approximately 3 times that of isooctane under these conditions. The vapor pressure of hexane is 3.0 times that of isooctane at 25°C (2).

Experiment 2. Evaporation Rates of Solvents from Glass-Stopper Volumetric Flasks

The average evaporation rates of hexane and isooctane from duplicate closed 10.0 mL volumetric flasks at ambient temperature (24°C) were established to be 0.15 (±0.07) mL/wk and 0.06 (±0.02) mL/wk, respectively. This difference is significant by Student's *t*-test at the 99% confidence limit. Under these conditions, the hexane evaporation rate is approximately 3 times that of isooctane.

A graph of evaporation rate vs boiling point temperature showed a direct relationship for all test solvents. However, because of the scatter on the plot, it was obvious that boiling point is not the only critical factor. A plot of solvent evaporation rate as a function of the ratio of vapor pressure to surface tension (D. Jernigan, Radian Corp., June 1979) gave a linear correlation coefficient of 0.9827 for the 8 test solvents.

Table 1 lists the experimental values obtained for the evaporation rate and the vapor pressure-to-surface tension ratios (2) of each of the solvents. In addition, there are vapor pressure-to-surface tension ratios listed for other solvents commonly used in the pesticide residue laboratory.

Table 1. Evaporation rates from 10 mL glass-stopper volumetric flasks and vapor pressure/surface tension ratios for some common organic solvents

Solvent	Vapor press./surf. tens. ratio,[a] torr cm/dyne	Evapn rate,[b] mL/wk ± SD
Pentane	33.1	—
Ethyl ether	32.4	0.63 ± 0.17
Methylene chloride	16.0	0.25 ± 0.10
Hexane	8.45	0.15 ± 0.07
Acetone	8.01	0.22 ± 0.01
Chloroform	7.32	—
Methanol	5.65	0.086 ± 0.17
Ethyl acetate	3.97	—
Acetonitrile	3.79	—
Benzene	3.38	0.096 ± 0.0013
Ethanol	2.73	—
Isooctane	2.68	0.058 ± 0.022
Heptane	2.31	—
Isopropanol	2.11	—
Toluene	1.02	0.045 ± 0.0021
Decane	0.04	—

[a] Calculated from Riddick and Burger (2).
[b] Only listed for this work.

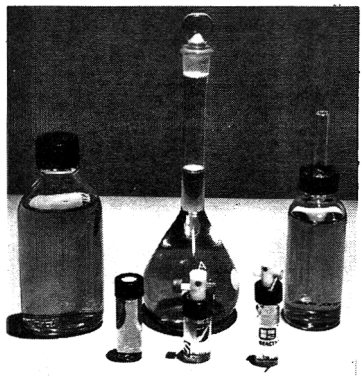

Figure 1. Test containers. Front row (l-r): 4 mL small vial, 0.3 and 1.0 mL conical vials. Back row (l-r): 3 oz prescription bottle, 100 mL volumetric flask, and 25 mL multivial (tested with the glass seal tube removed and closed with the vial cap).

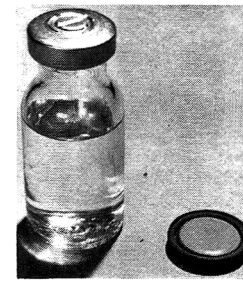

Figure 2. Serum bottle (10 mL) and Teflon-faced septum. Note puncture hole in septum on right after one puncture with standard beveled-point needle from 10 μL Hamilton syringe.

Wrapping Teflon plumber's tape on the outside of the joint between glass stoppers and the neck of the volumetric flask did not reduce the evaporation rate of the solvent inside the container.

Experiment 3. Solution Volume Effects for Glass-Stopper Containers

Four sizes of volumetric flasks were tested in duplicate at ambient temperature to evaluate the effect of solution volume on the solvent evaporation rate. The average evaporation rates of hexane from 5, 10, 50, and 100 mL volumetric flasks were 0.16 (±0.03), 0.15 (±0.07), 0.11 (±0.06), and 0.11 (±0.04) mL/wk, respectively. The decreasing evaporation rates with increasing solution volume in itself makes the larger volume solutions somewhat more desirable. But, more important, the relative evaporation rates (percent of the solution volume evaporated per week) were 3.3, 1.5, 0.23, and 0.10% of the container volume evaporated per week, respectively. Use of larger flasks clearly increases the useful life-time of the reference standards

Experiment 4. Evaporation Rates of Solvents at Different Storage Temperatures

Average evaporation rate for 10.0 mL glass-stopper volumetric flasks filled with hexane and stored in duplicate at ambient temperature (23–24°C), in the refrigerator (+4°C), and in the freezer (−15°C) were 0.15 (±0.07), 0.06 (±0.12), and 0.02 (±0.001) mL/wk, respectively. The rate of loss at refrigerator temperature was approximately a factor of 3 less than rate of loss at ambient. At freezer temperature, the factor was approximately 7 less than ambient. The simple precaution of refrigerator or preferably freezer storage will significantly increase the useful life of standard solutions.

Experiment 5. Evaporation Rates of Solvents from Different Containers

The evaporation rates of isooctane and hexane were determined at ambient temperature for all sizes of glassware listed under *Reagents and Equipment* (Figures 1 and 2).

In Figure 3, the horizontal lines represent the range of the evaporation rates for the solvent/glassware-type combinations. The vertical tick marks on the horizontal lines represent the average evaporation rates calculated for each of the tested sizes of the glassware. In the case of the small vials, the vertical tick marks represent the tested vial size/vial closure combinations used in the experiment.

Volumetric Flasks.—The results in Figure 3 give ample illustration of the fast evaporation rate of hexane and the significantly lower evaporation rate of isooctane from closed volumetric flasks. Care should be taken when closing the flask. A small twist of the stopper greatly improves the seal. When more than 3–5% of the solvent is evaporated, the standard solution should be replaced.

Prescription Bottles.—As seen in Figure 3, the evaporation rates of solvent (both hexane and isooctane) from prescription bottles are somewhat lower than evaporation rates of isooctane from volumetric flasks. The evaporation rate range of the 2 solvents from prescription bottles is 0.01–0.03 mL/wk. These evaporation rates are low enough to keep solvent loss under control from a 50 mL volume. As in the evaporation of solvent from volumetric flasks, firmly tightening

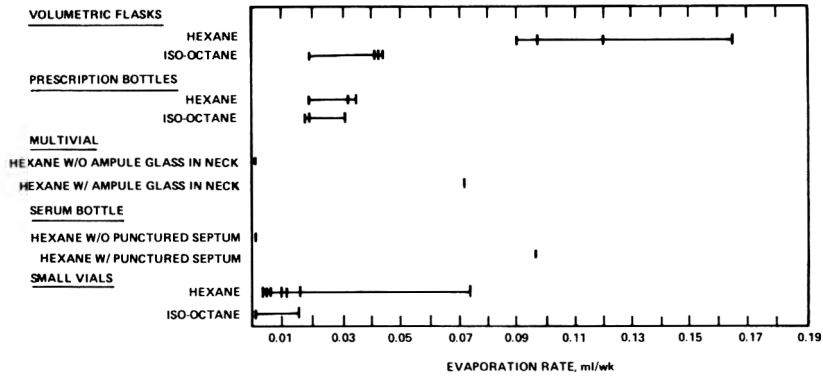

Figure 3. Comparative evaporation rates (mL/wk) of hexane and/or isooctane from different containers at ambient temperature. Range of data for each container is plotted. Vertical lines represent different container volumes (i.e., volumetric flasks and prescription bottles) or different container/cap configurations (i.e., small vials).

the cap on a prescription bottle helps to reduce the solvent evaporation rate. Caps with a hard liner backing underneath the Teflon liner give the best seal. Caps with soft foam-backed seals should be avoided, because they can be totally distorted away from the mouth of the bottle when tightened.

Multivials, Septum Bottles, and Small Vials.—We discourage the use of these containers because of handling problems and/or unacceptable solvent evaporation rates. The solvent evaporation rate from multivials with the ampule top removed, using the container as a screw-cap vial, is almost double the worst case for prescription bottles (0.07 mL/week) unless the glass below the snap-off score line is removed with a punch or pliers. When the glass below the snap-off score line is not removed, protruding sharp edges tear the septum cap seal, resulting in contamination of the solution in the vial. The serum bottle (Figure 2) solvent evaporation rate is almost triple the prescription bottle rate (0.09 mL/wk) *after* the bottle seal has been punctured with the beveled needle of a standard 10 μL Hamilton syringe. The relative evaporation rates from all of the small vials tested (Figure 1) are all greater than 5% of the solution per week, making them useless for long-term storage of standards.

General Considerations

A great deal of caution must be used in the interpretation of the values of the evaporation rates presented in this paper. The evaporation rates presented are a function of within-lot and between-lot container closure variations as well as care taken by the user in firmly sealing the closure. This variability is evident in the low experimental precision in the values reported. Efforts on our part to obtain the same evaporation rates, when the work was performed by another chemist, were unsuccessful. The differences between the evaporation rates of solvents from the various containers proved to be consistent between experimenters, thereby confirming experimental conclusions. Laboratories are also cautioned against applying the same experimental conclusions to similar glassware but of different design or manufacture. The analyst is encouraged to perform his own evaluation of solvent containers which are not specifically covered by this investigation.

Conclusions and Recommendations

The results of this study clearly demonstrate that solvent evaporation from organic reference standard solutions is a significant source of error that must be controlled by the analyst. Solvent evaporation can only be minimized by the proper handling and storage of all organic solutions. From the results of this study and good QA practices, the following general recommendations are made to help minimize solvent evaporation:

(*1*) Use the solvent with the lowest possible evaporation rate or vapor pressure-to-surface tension ratio that will dissolve the material. Isooctane, toluene, and other higher molecular weight hydrocarbons are recommended for the lower polarity pesticides.

(*2*) Choose a container of approximately 100 mL volume, such as prescription bottles or volumetric flasks, for the storage of often-used ref-

erence standard solutions. Alternatively, small volumes may be stored if the solution is divided into several small containers (about 20 mL) and kept in a refrigerator or freezer. They may be withdrawn one at a time, used for a maximum of one week, and then discarded.

(3) Mark the solvent level when the container is originally filled with the solution. Monitor the solvent level in the container. Aliquots of the final dilution (working solution) should not be removed for other uses because then it is impossible to know if solvent has evaporated.

(4) Store solutions that are not in daily use in a refrigerator or freezer to reduce solvent losses.

(5) Seal containers tightly to reduce evaporation from around the cap seal.

(6) If in doubt about solution concentration beyond 3–5%, either check the solution against a solution known to be accurate or replace the standard with a freshly prepared standard.

(7) Do not store standard solutions for more than one year.

Acknowledgments

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References

(1) Hodgson, D. W., Thompson, J. F., & Watts, R. R. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 94–102

(2) Riddick, J. A., & Burger, W. B. (1970) "Organic Solvents," in *Techniques of Chemistry*, Vol. 2, A. Weissberger (Ed.), Wiley-Interscience, New York, NY

Accuracy of Pesticide Reference Standard Solutions. Part II. Chemical Stability Under Four Storage Conditions

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A study was undertaken to assess the long-term chemical stability of dilute standard pesticide solutions of 4 compound classes. The solutions were studied under 4 storage conditions: freezer at −15°C; refrigerator at 3°C; ambient temperature in the dark; and ambient temperature on the bench top exposed to fluorescent and natural light. Pesticide compounds including 27 organochlorines, 20 organophosphates, 10 triazines, and 13 carbamates were monitored for periods of 6 months to 2 years. Most of the compounds were stable (<10% decomposition) under all conditions for the full length of the study. Solutions of CDEC, butylate, and disulfoton, however, decomposed when stored under all storage conditions. Solutions of carbaryl, methiocarb, and carbofuran decomposed when stored at ambient laboratory temperature with exposure to light. Recommendations are made for formulating solvents, storage, and practical shelf life of high concentration stock solutions and dilute gas chromatographic working standards representing 4 pesticide classes.

A careful consideration of the integrity of standard solutions is of utmost importance to any analytical laboratory. Pesticide residue chemists are generally concerned with the concentration accuracy and chemical stability of their reference solutions. However, laboratory time and resources are rarely available for conducting the long-term studies necessary to evaluate procedures for preparation, storage maintenance, and use of standard solutions.

Suett et al. (1) reported on a 2-year evaluation of the storage stability of 6 pesticides after our study had been completed. The compounds were formulated in 3 solvents at 1 mg/mL, sealed in ampules, and stored in the dark at −20, 1, and 20°C. The study concluded that these concentrated stock solutions were at least as stable as the pure primary standards from which they were prepared.

A reference standard solution stability study was also reported by the Food and Drug Administration (2) for 7 common organochlorine pesticides. Materials were formulated in isooctane and stored in Pyrex volumetric flasks at ambient temperature with exposure to fluorescent lamps. It was concluded that no qualitative changes occurred in the dilute solutions during the 8-month testing period. The experiment was not designed to monitor changes in concentration.

Investigations were conducted by this quality assurance coordinating laboratory, which resulted in a 2-part report of the various factors affecting the analytical integrity of standard solutions representing 4 pesticide classes. The first study (3) concerned the effect of the choice of solvent, solution container, and storage conditions on the evaporation of the solvent and the resultant changes of the standard solution concentration.

The present report describes studies undertaken to assess the long-term chemical stability of standard reference materials which have been formulated in appropriate organic solvents and stored under various conditions. This study encompasses 2 areas of investigation. The first is an evaluation of chemical stability of dilute gas chromatographic (GC) standard reference solutions (working solutions) of organochlorine, organophosphate, carbamate, and triazine pesticides in solvents suitable for dissolution of the compounds and also compatible with the GC detector systems. The second and similar evaluation was conducted on acetone solutions of organochlorine and organophosphate pesticides formulated for use as fortification solutions in our quality assurance program for water and blood serum analyses.

These 2 stability studies were intended to identify compounds that are not stable in solution, and to evaluate the magnitude of the instability under normal residue laboratory conditions. No effort was made to rigorously control the temperatures, evaluate the total amount of light radiation absorbed by the test solutions, or in any other way to make this a rigorous physical-chemical study. The figures presented in this paper are presented as representations of the data trend. They are not intended to be

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.
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studied in great detail to establish the degradation curve of each specific compound.

Experimental

Reagents and Materials

(a) *Solvents.*—Isooctane (2,2,4-trimethylpentane), hexane, toluene, ethyl acetate, and acetone; all distilled-in-glass quality (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

(b) *Analytical standards.*—Obtained from the Pesticides Analytical Standards Repository, Environmental Protection Agency, Research Triangle Park, NC 27711. (*1*) *Organochlorines.*—Aldrin (99.5%), Aroclor 1016, Aroclor 1254, β-BHC (99.8%), technical chlordane, chlordecone (87.3%), chlordene (99.6%), DCPA (99.9%), *p,p'*-DDD (99+%), *p,p'*-DDE (99%), *o,p'*--DDT (99+%), *p,p'*-DDT (99+%), dieldrin (99.9%), endosulfan I (100%), endosulfan II (100%), endrin (99.0%), hexachlorobenzene (98+%), heptachlor (99.8%), heptachlor epoxide (99.3%), 1-hydroxychlordene (100%), lindane (99.9%), methoxychlor (99+%), mirex (99.9%), *trans*-nonachlor (100%), oxychlordane (98%), PCNB (99.03%), and toxaphene (68.9% Cl).

(*2*) *Organophosphates.*—Azinphos methyl (99.5%), carbophenothion (98.5%), chlorpyrifos (99%), DEF (94.6%), dichlofenthion (97.0%), dimethoate (99.5%), dioxathion (74%), disulfoton (98.8%), ethion (95.5%), ethoprop (99%), fenthion (98.2%), leptophos (100%), malathion (99.3%), methyl parathion (99+%), mevinphos (64.5%), parathion (99+%), phencapton (98.5%), phorate (99.9%), phosmet (99.5%), and ronnel (99+%).

(*3*) *Carbamates.*—Aminocarb (97.0%), bufencarb (97.8%), butylate (99.7%), carbaryl (99.7%), carbofuran (99.5%), CDEC (98.6%), mecarbam (99.6%), methiocarb (99.0%), mexacarbate (99%), pirimicarb (100%), promecarb (>98%), propoxur (98.4%), and thiobencarb (99.9%).

(*4*) *Triazines.*—Ametryn (99+%), atraton (89+%), atrazine (99+%), cyanazine (99%), cyprazine (99.3%), prometon (99.5%), prometryn (99+%), propazine (99+%), simazine (99+%), and terbutryn (98+%).

(c) *Standard prescription bottles.*—Clear glass, screw-cap, 2 oz and ½ oz (Brockway Glass Co. Inc., Brockway, PA).

(d) *Teflon cap liners.*—For prescription bottles (A. H. Thomas, Philadelphia, PA; Cat. No. 2390-H).

(e) *Gas chromatographs.*—(*1*) Tracor Model 222 equipped with ^{63}Ni electron capture detectors (ECD) operated in the DC mode and dual flame photometric detector (FPD); 1.8 m × 4 mm id glass columns for ECD packed with 1.5% OV-17/1.95% OV-210 on 80–100 mesh Gas-Chrom Q; 1.8 m × 4 mm id glass column for FPD packed with 4% SE-30/6% OV-210 on 80–100 mesh Gas-Chrom Q; oven 190–200°C; injector and transfer line 250°C; column nitrogen flow 60 mL/min; FPD gases 50 mL hydrogen/min and 100 mL air/min.

(*2*) Hewlett-Packard Model 5710 equipped with N/P thermionic detectors (NPD); 1.8 m × 4 mm id glass column packed with 5% OV-101 on 100–120 mesh Carbowax 20M support-bonded on Chromosorb WAW (4, 5); oven 180–200°C; injector 200°C; detector 300°C; detector gas flows, 3 mL hydrogen and 50 mL air/min; column gas flow 60 mL helium/min.

Experimental Procedure

The following definitions apply for this experiment:

Primary standard: the pure standard compound as received from the Standards Repository.

Stock solutions: solutions of individual compounds (concentration >100 μg/mL) prepared directly from primary standards, used to prepare solutions of individual compounds or mixtures at appropriate injection concentrations, and used as a reference point in the experiment.

Working solutions: solutions at the concentration appropriate for injection (ECD <100 pg/μL, FPD and NPD between 100 pg/μL and 10 ng/μL) and tested in this experiment.

In this experiment, one month is defined as 28 days.

All quantitations were done by manual peak height measurement.

Reference Stock Solution Preparation and Storage for Both Studies

On the first day of the experiment, reference stock solutions were prepared from the respective primary standards at approximately 200 μg/mL. The stock solutions of the organochlorines and organophosphates were prepared in benzene. Carbamate stock solutions were prepared in toluene. Stock solutions of triazines were prepared in ethyl acetate because of insolubility in toluene and benzene. All stock solutions were stored in the freezer at −15°C when not in use.

Preparation, Storage, and Stability Testing of Dilute Working Solutions

Organochlorines and organophosphates (*in isooc-*

Table 1. Organochlorine and organophosphate isooctane solutions and 2-year stability test results

Compound	Soln[a]	Concn, pg/μL	Degradation at storage condition[b]			
			Freezer −15°C	Refrig. +3°C	Dark ~24°C	Light ~24°C
Organochlorines						
Aldrin	B	9.9	+	+	+	+
Aroclor 1016	E	100	+	+	+	+
Aroclor 1254	F	200	+	+	+	+
β-BHC	C	16.2	+	+	+	+
γ-BHC (lindane)	C	6.0	+	+	+	+
Chlordane (tech)	G	80.4	+	+	+	+
Chlordecone[c]	K	19.5	+	+	+	+
Chlordene	D	5.3	+	+	+	+
DCPA	B	15.1	+	+	+	+
p,p'-DDD	B	30.2	+	+	+	+
p,p'-DDE	A	22.0	+	+	+	+
o,p'-DDT	A	36.5	+	+	+	+
p,p'-DDT	A	47.8	+	+	+	+
Dieldrin	B	20.6	+	+	+	+
Endosulfan I	C	26.0	+	+	+	+
Endosulfan II	C	30.0	+	+	+	+
Endrin	C	50.0	+	+	+	+
HCB	C	5.0	+	+	+	+
Heptachlor	A	8.9	+	+	+	+
Heptachlor epoxide	A	15.0	+	+	+	+
1-Hydroxychlordene	D	15.8	+	+	+	+
Methoxychlor	F	71.4	+	+	+	+
Mirex	A	48.3	+	+	+	+
trans-Nonachlor	D	14.0	+	+	+	+
Oxychlordane	D	13.7	+	+	+	+
PCNB	A	7.8	+	+	+	+
Toxaphene	H	452	+	+	+	+
Organophosphates						
Azinphos methyl	J	2500	+	+	+	+
Carbophenothion	J	400	+	+	+	+
Chlorpyrifos	J	150	+	+	+	+
DEF	J	300	+	+	+	+
Dichlofenthion	K	100	+	+	+	+
Dimethoate	L	100	+	+	+	+
Dioxathion	L	300	+	+	+	+
Disulfoton	J	75	−(13.8%)	+	−(34.2%)	−(68.8%)
Ethion	K	150	+	+	+	+
Ethoprop	L	50	+	+	+	+
Ethyl parathion	K	150	+	+	+	+
Fenthion	L	100	+	+	+	+
Leptophos	K	850	+	+	+	+
Malathion	L	200	+	+	+	+
Methyl parathion	K	100	+	+	+	+
Mevinphos	L	100	+	+	+	+
Phencapton	K	130	+	+	+	+
Phorate	J	50	+	+	+	+
Phosmet	J	1250	+	+	+	+
Ronnel	K	100	+	+	+	+

[a] Identifies components of pesticide mixtures.
[b] Stable (+) or unstable (−) and % decomposition.
[c] Solvent 1% methanol in benzene because of electron capture response characteristics (4).

tane).—To reduce the number of necessary GC injections, mixtures of structurally similar standards were prepared by dilution to appropriate injection level concentrations. The mixture compositions and concentrations are specified in Table 1. Each injection concentration working standard solution was divided into three ½ oz prescription bottles with Teflon-lined caps and stored as follows: (*1*) one in the freezer (−15°C) for one year; (*2*) one in the refrigerator (+3°C) for one year; and (*3*) one at ambient laboratory temperature (ca 25°C) stored in the cabinet under the laboratory bench without exposure to light for one year, followed by storage at

Table 2. Carbamate toluene solutions and triazine ethyl acetate solutions and 1-year stability test results

Compound	Concn, pg/μL	Degradation at storage condition[a] Freezer −15°C	Refrig. +3°C	Dark ~24°C	Light ~24°C
Carbamates (in Toluene)					
Aminocarb	200	+	+	+	+
Bufencarb	800	+	+	+	+
Butylate	200	−(51%)	−(51%)	−(51%)	−(49%)
Carbaryl	1600	+	+	+	−(38%)
Carbofuran	400	+	+	+	−(17%)
CDEC	30	−(65%)	−(59%)	−(47%)	−(98%)
Mecarbam	100	+	+	+	+
Methiocarb	400	+	+	−(13%)	−(40%)
Mexacarbate	300	+	+	+	+
Pirimicarb	120	+	+	+	+
Promecarb	400	+	+	+	+
Propoxur	200	+	+	+	+
Thiobencarb	400	+	+	+	+
Triazines (in Ethyl Acetate)					
Ametryn	400	+	+	+	−(17%)
Atraton	200	+	+	−(14%)	+[b]
Atrazine	200	+	+	−(50%)	−(13%)
Cyanazine	500	+	+	+	+
Cyprazine	200	+	+	+	+
Prometon	200	+	+	+	−(11%)
Prometryn	300	+	+	+	−(11%)
Propazine	200	+	+	+	+
Simazine	200	+	+	+	+
Terbutryn	400	+	+	+	+

[a] Stable (+) or unstable (−) and % decomposition.
[b] Reference to discussion, this solution concentrated.

ambient temperature on the laboratory bench exposed to fluorescent and natural light for another year.

The 3 storage test solutions were compared with fresh dilutions of the respective reference stock solutions monthly (28 days) to monitor for changes in the concentration of any of the compounds and to watch for the appearance of any decomposition products. The test solutions and the fresh reference standard solutions were always injected in duplicate.

Fresh re-weighed reference stock solutions of each compound were prepared every 3–4 months from the original primary standard used on day 1. After establishing that the new reference standard solutions agreed within 3% of old reference standard solutions, the older reference solutions were discarded. All analyses of the respective organochlorine and organophosphate mixtures were made on the Tracor 222 gas chromatograph. The electron capture detector was used for the organochlorines and the flame photometric detector (P mode) was used for the organophosphates.

Carbamates (in toluene) and triazines (in ethyl acetate).—The experimental design was identical to that of the organochlorines and organophosphates with 2 modifications. Because of smaller numbers of compounds in these 2 groups and suspected catalytic breakdown of carbamates in solution as mixtures (R. Moseman, Environmental Protection Agency, Research Triangle Park, NC; February 1975), these test solutions were formulated individually. Three samples of each test solution in ½ oz prescription bottles were prepared and stored at the same conditions used for organochlorines and organophosphates. A fourth test solution was stored at ambient temperature exposed to light. Table 2 lists the compounds tested and their respective solution concentrations.

All GC analyses of these 2 classes were made on the Hewlett-Packard 5710 gas chromatograph equipped with N/P thermionic detectors. The modified Carbowax 20M support-bonded column packings (4, 5) were used because of their vastly improved chromatography of carbamate compounds (6).

Table 3. Organochlorine and organophosphate acetone solutions and half-year stability test results

Compound	Soln[a]	Concn, pg/μL	Degradation at storage condition[b] Freezer −15°C	Refrig. +3°C	Dark ~24°C	Light ~24°C
Organochlorines						
Aldrin	N	100	+	+	+	+
Aroclor 1016	O	1000	+	+	+	+
Aroclor 1254	P	2000	+	+	+	+
β-BHC	M	160	+	+	+	+
Chlordane (tech)	Q	805	+	+	+	+
p,p'-DDE	M	220	+	+	+	+
p,p'-DDT	M	475	+	+	+	+
Dieldrin	N	200	+	+	+	+
Endrin	M	660	+	+	+	+
HCB	M	50	+	+	+	+
Heptachlor	N	90	+	+	+	+
Heptachlor epoxide	N	150	+	+	+	+
1-Hydroxychlordene	N	150	+	+	+	+
Organophosphates						
Carbophenothion	R	4000	+	+	+	+
Chlorpyrifos	R	2000	+	+	+	+
Dichlofenthion	R	1000	+	+	+	+
Dimethoate	T	5000	+	+	+	+
Ethion	S	2500	+	+	+	+
Ethyl parathion	R	1750	+	+	+	+
Fenthion	S	1500	+	+	+	+
Mevinphos	T	1000	+	+	+	+
Phorate	R	500	+	+	+	+
Ronnel	R	1250	+	+	+	+

[a] Identifies components of pesticide mixtures.
[b] + = stable.

Preparation, Storage, and Stability Testing of Acetone Formulation Solutions

Organochlorines and organophosphates.—On the first day of the experiment, stock solutions were prepared in benzene as previously mentioned. Dilute mixtures were then prepared in acetone at concentrations 10 times that normally used for GC injections (Table 3). These mixtures were subdivided into 2 different storage containers for solvent evaporation comparison: 6 sealed glass ampules (one per month of the study) and a prescription bottle. All samples were stored at −15°C when not in use. Solutions from both container types were monitored monthly. Acetone mixtures were diluted 1:10 with isooctane just before GC quantitation. (A study conducted at the beginning of the experiment indicated that the detector response was not affected by the presence of 10% acetone in isooctane.) The electron capture detector was used for organochlorines; organophosphates were analyzed with the Tracor 222 equipped with a flame photometric detector (P mode).

Because this acetone study was initiated after completion of the GC working reference standard solution study, the reference solutions of organochlorines and organophosphates were known to be stable for up to one year when stored in the freezer at −15°C. Reference standards for this study were, therefore, prepared at injection concentration as isooctane dilutions of the reference stock solutions and stored in the freezer.

Results and Discussion

By the criterion that decomposition of less than 10% over the length of the study is defined as stable, 59 of 70 compounds tested proved to be stable in the working solution study. Eight compounds were unstable only at ambient temperature and 3 other compounds were unstable under all storage conditions. All of the 13 organochlorines and 10 organophosphates tested in the acetone formulation solution study proved to be stable in the freezer by this criterion.

Working Standards Stability Experiment

Organochlorines (in Isooctane).—All compounds

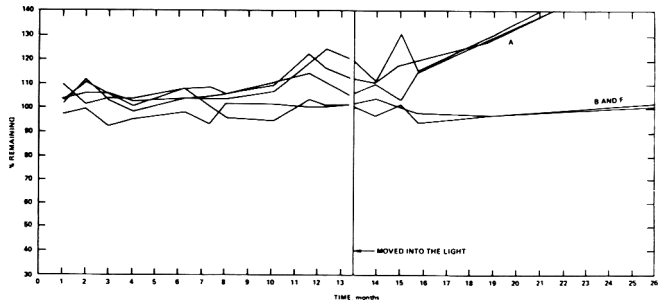

Figure 1. Degradation curves of DDT-type compounds from working standard study (isooctane, ambient temperature). Degradation curves from solution A (*p,p'*-DDT, *p,p'*-DDE, and o,*p'*-DDT) illustrate solution concentration by solvent evaporation. B = *p,p'*-DDD; F = methoxychlor.

in this class (24 single component compounds, plus toxaphene, chlordane, and Aroclors 1016 and 1254) were stable in isooctane under test conditions during the 2 years of study (Table 1). The compounds were stable to chemical degradation even under the most severe test conditions. As illustrated in Figures 1 and 2, the stability data plots of the DDT-type compounds and the Aroclors do not indicate any reduction of concentration. The 3 compounds from solution A increased in concentration after month 10, obviously due to solvent evaporation because only two-thirds of the solvent was left at the end of 2 years. The other 2 compounds are of the same general chemical structure (*p,p'*-DDD and methoxychlor), but were in mixtures B and F that did not exhibit concentration through solvent evaporation.

Only a few of the compounds could be illustrated, but they are typical of all of the other compounds tested.

Organophosphates (in Isooctane).—All compounds in this class (20 compounds) except disulfoton showed no degradation in isooctane during 2 years under all test conditions (Table 1; Figures 3 and 4). The degradation of disulfoton was evident under all storage conditions (Figure 4). The results illustrated, with the exception of disulfoton, are typical of the remaining organophosphates tested.

Carbamates (in Toluene).—Only 8 of the 13 test compounds in this class were stable in toluene under all 4 test conditions (Table 2). CDEC (in isooctane) and butylate decomposed rapidly under all test conditions (Figure 5). Butylate decomposed approximately 50% per year under all test conditions. CDEC decomposed 50–98% per year depending on storage conditions.

Carbaryl, carbofuran, and methiocarb decomposed 38, 17, and 40%, respectively, with exposure to light at ambient laboratory temperature in one year. Of these, only methiocarb was unstable (13% decomposition) during the same time period at ambient temperature when stored in the dark. Solutions of aminocarb, thiobencarb, mecarbam, bufencarb, mexacarbate, pirmicarb, promecarb, and propoxur were stable for one year under all conditions tested. Representative examples of some of the ambient-dark degradation curves are given in Figure 6.

Triazines (in Ethyl Acetate).—Only half the compounds tested in this group were stable

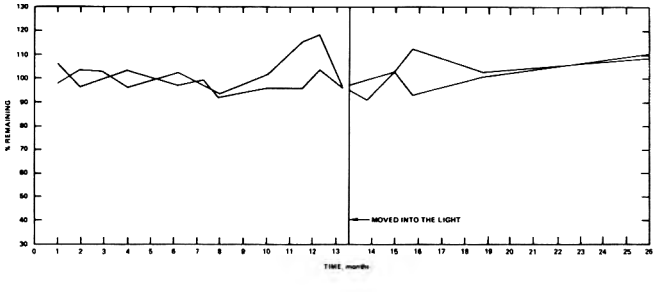

Figure 2. Degradation curves of Aroclors 1016 and 1254 from working standard study (isooctane, ambient temperature).

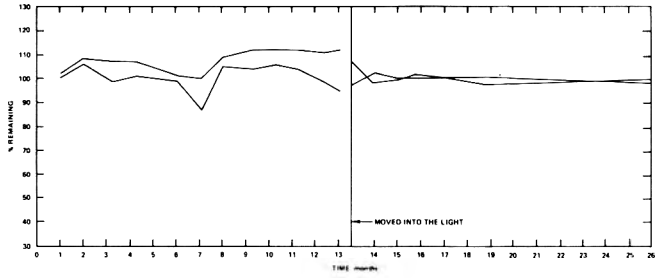

Figure 3. Degradation curves of ethyl and methyl parathion from working standard study (isooctane, ambient temperature).

under all test conditions (Table 2). Figures 7 and 8 are representative of the stability curves of some stable compounds: chlorine-, methoxy-, and thiomethyl-substituted compounds.

Atrazine and atraton degraded approximately 15% in one year at ambient temperature in the dark. Prometryn, prometon, atrazine, and ametryn degraded 11-17% at ambient temperature with exposure to light. Because of solvent evaporation from the atraton solution container, decomposition of atraton with light exposure could not be measured.

Acetone Formulations of Organochlorines and Organophosphates

All 19 individual compounds and 3 technical mixtures (chlordane and Aroclors 1016 and 1254) in acetone were stable at −15°C to chemical degradation for the 6 months of the test (Table 3) and would be suitable for preparation of quality assurance samples. No evaporation was evident in either the ampule or the screw-cap prescription bottle.

Degradation Curve

Because of the extent of chemical breakdown of disulfoton, CDEC, and butylate, their data were used as models for regression analysis. The regression correlation coefficients for the first order rate equation

$$\ln \frac{C_0}{C} = kt, \quad \text{or} \quad \frac{C}{C_0} = e^{-kt}$$

were high (>95% confidence), indicating that degradation of these compounds was at least pseudo-first order. The calculated time necessary for 10% decomposition of the unstable compounds is listed in Table 4.

Degradation rate curves show exponential decreases in concentration. The most severe changes, therefore, occur early in the life of the standard solution. General recommendations reflect this fact and consequently may appear more conservative than a cursory review of results would indicate.

Conclusions and Recommendations

The majority of compounds proved to be stable (less than 10% concentration change) in organic solution for one year when stored under any of the 4 test conditions. Therefore, standard solutions of these stable compounds can be used for one year for accurate quantitative determinations as long as solvent evaporation is properly con-

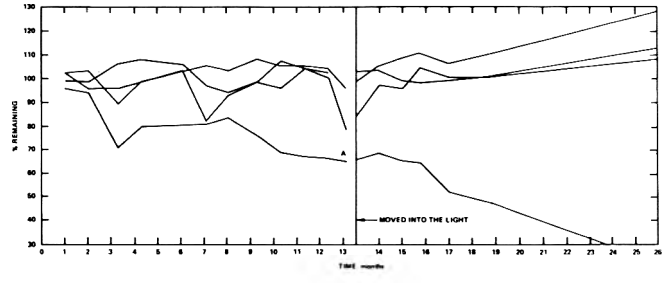

Figure 4. Degradation curves of organophosphates containing thio-ether functions from working standard study (isooctane, ambient temperature). Compounds include phencapton, fenthion, carbophenothion, and disulfoton; disulfoton (A) illustrates significant degradation.

Table 4. Time (in months) for 10% degradation of unstable compounds under test conditions

Compound	Conditions			
	Freezer −15°C	Refrig. +3°C	Dark ~24°C	Light ~24°C
Organophosphates (in isooctane)				
Disulfoton	2.18	—[a]	3.27	1.18
Carbamates (in toluene)				
Butylate	2.02	1.90	1.91	1.93
Carbaryl	—	—	—	2.55
Carbofuran	—	—	—	7.58
CDEC	1.31	1.54	2.13	0.368
Methiocarb	—	—	10.2	2.70
Triazines (in ethyl acetate)				
Ametryn	—	—	—	7.21
Atraton	—	—	8.93	—[b]
Atrazine	—	—	2.17	9.66
Prometon	—	—	—	11.3
Prometryn	—	—	—	11.7

[a] <10% degradation.
[b] Reference to discussion, this solution concentrated.

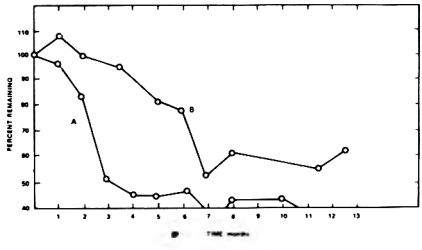

Figure 5. Degradation curves of CDEC in isooctane (A) and butylate in toluene (B) from working standard study (ambient temperature in the dark).

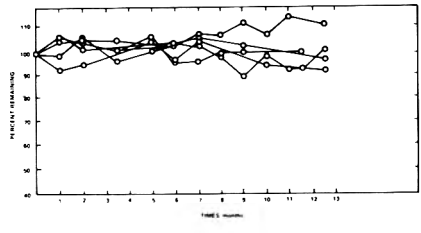

Figure 6. Degradation curves of 5 aromatic *N*-methyl carbamates from working standard study (toluene, ambient temperature in the dark). Compounds include propoxur, carbofuran, methiocarb, carbaryl, and aminocarb.

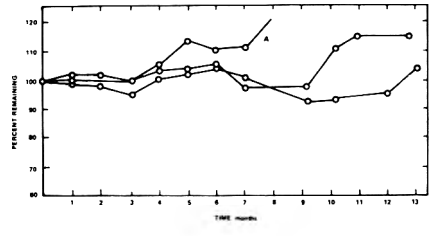

Figure 7. Degradation curves of triazines containing chlorine on the ring from working standard study (toluene, ambient temperature in the dark). Compounds include simazine, cyanazine, and propazine. Simazine (A) illustrates solvent evaporation after month 4.

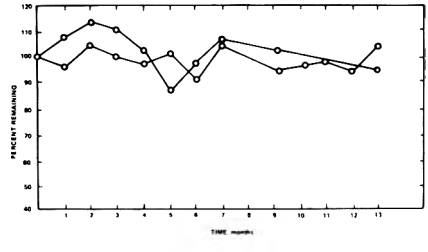

Figure 8. Degradation curves of triazines containing $-OCH_3$ and $-SCH_3$ on the ring from working standard study (toluene, ambient temperature in the dark). Compounds include ametryn and atraton.

trolled through use of recommended solvents, storage vessels, storage conditions, and solvent volume monitoring (see Part I, ref. 3). Acetone solutions of organochlorine and organophosphate pesticides were also useful for at least 6 months.

Quality control practices recommended from the stability study results are as follows:

(1) Primary reference standards: Store in a desiccated container in the freezer.

(2) Concentrated stock solutions: Store in a freezer. Every year, replace compounds that did

not degrade (Tables 1 and 2). Every 6 months, replace compounds that degrade only at ambient temperature. This group includes carbaryl, carbofuran, methiocarb, ametryn, atraton, atrazine, prometon, and prometryn. Monthly, replace compounds that degraded under all test conditions. This group includes disulfoton, CDEC, and butylate. Other alkyl carbamates that were not tested, such as vernolate, pebulate, etc., should probably also be in this group.

(3) Dilute working solutions: Store stable compounds (Tables 1 and 2) in a refrigerator if not in daily use. Replace by dilution from stock solution every 6 months. If solutions were used often and left on bench top, replace by dilution from stock solution every 3 months. Replace sooner if evaporation is evident. Compounds known to be unstable at ambient temperature should be stored in a refrigerator between uses and be replaced with fresh dilutions from stock solutions every 2 months. Replace sooner if evaporation is evident. Unstable compounds should be stored in a refrigerator between uses and be replaced with fresh dilutions from stock solutions every week.

Acknowledgments

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References

(1) Suett, D. L., Wheatley, G. A., & Padbury, C. E. (1979) *Analyst* **104,** 1176–1180
(2) *Pesticide Analytical Manual* (1979) Vol. 1, Section 133, Food and Drug Administration, Washington, DC
(3) Hodgson, D. W., & Watts, R. R. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 89–93
(4) Moseman, R. F. (1978) *J. Chromatogr.* **166,** 397–402
(5) Hall, R. C., & Harris, D. E. (1979) *J. Chromatogr.* **169,** 245–259
(6) Hodgson, D. W., Kantor, E. J., & Mann, J. B. (1978) *Arch. Environ. Contam. Toxicol.* **7,** 99

COLOR ADDITIVES

Automated High Performance Liquid Chromatographic Determination of Intermediates and Side Reaction Products in FD&C Red No. 3

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A rapid, sensitive, reproducible method that uses high performance liquid chromatography in the reverse phase mode is described for the determination of intermediates and side reaction products in FD&C Red No. 3. With this method, 2 intermediates, phthalic acid and resorcinol, and 2 side reaction products, 2-(2',4'-dihydroxybenzoyl)benzoic acid and 2-(2',4'-dihydroxy-3',5'-diiodobenzoyl)benzoic acid, are eluted in a reproducible pattern as a gradient increases the organic nature of the buffered mobile phase. Phthalic acid and the 2 side reaction products are quantitated. The intermediate resorcinol is detected but not quantitated. Recoveries averaged 99.4-102.8% for phthalic acid and the 2 side reaction products added to samples of FD&C Red No. 3 at levels ranging from 0.02 to 0.22%.

FD&C Red No. 3 (Colour Index No. 45430) is a synthetic color additive that is prepared by condensation of resorcinol with phthalic anhydride to produce fluorescein (Figure 1) which in turn is iodinated and treated with NaOH to produce FD&C Red No. 3 (Figure 2). Every batch of certifiable FD&C Red No. 3 that is manufactured must first be chemically analyzed and approved by the Certification Branch, Division of Color Technology, Food and Drug Administration (FDA) before it can be used in a food, drug, or cosmetic in the United States. The Code of Federal Regulations (CFR) (1) limits the amounts of the intermediates, resorcinol and phthalic acid, to a total of 0.1% by weight of FD&C Red No. 3. The limits for 2-(2',4'-dihydroxy-3',5'-diiodobenzoyl)benzoic acid and 2,4,6-triiodoresorcinol are each 0.2% by weight of FD&C Red No. 3. The CFR imposes no specific limit on the amount of 2-(2',4'-dihydroxybenzoyl)benzoic acid that may be present in FD&C Red No. 3. The CFR states, however, that a limit for this side reaction product may be set in accordance with good manufacturing practice.

In an internal progress report, Graichen and Watkins reported that 2,4,6-triiodoresorcinol arises from the iodination of resorcinol (C. Graichen and N. Watkins, Division of Color Technology, 1965). They also reported that when 2,4,6-triiodoresorcinol and FD&C Red No. 3 are mixed together as powders, less than 1% of the 2,4,6-triiodoresorcinol added is recovered 6 days later. We prepared solutions containing mono-, di-, and triiodinated resorcinols. After 4 h these solutions had decomposed to resorcinol and monoiodoresorcinol. Therefore we made no attempt to determine or recover triiodoresorcinol.

At the present time, the intermediates in FD&C Red No. 3 are being determined by a cellulose column/ammonium sulfate eluant procedure (2). This method gives reproducible results, but is time-consuming and limited in its ability to resolve contaminants that might be found in commercial samples of this dye. A new reverse phase high performance liquid chromatographic (HPLC) method using a Zorbax C-8 column has been developed which separates all known contaminants, is faster, is fully automated, and gives greater reproducibility of results. The intermediate phthalic acid and the side reaction products 2-(2',4'-dihydroxybenzoyl)benzoic acid and 2-(2',4'-dihydroxy-3',5'-diiodobenzoyl)benzoic acid are quantitated; the intermediate resorcinol, which has never been found in a sample of FD&C Red No. 3 submitted for certification, is detected but not quantitated.

METHOD

Apparatus

(a) *Liquid chromatograph.*—With gradient elution capability. Altex Model 420 with 2 Altex Model 110-A pumps (Altex Scientific Inc., Berkeley, CA 94710), or equivalent. Operating conditions: chart speed 0.2 in./min; flow rate 1.0 mL/min; column temperature ambient. Gradient program: from 20 to 65% B in 12 min; from 65 to 85% B in 18 min; from 85 to 100% B in 5 min; hold at 100% B for 3 min; from 100 to 20% B in 4

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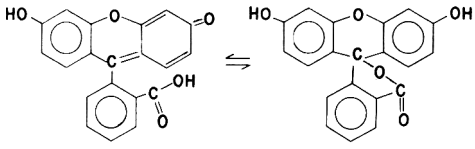

Figure 1. Fluorescein.

min; then hold at 20% B for 3 min and start injection program. Injection program: Signal is sent to autoinjector to rinse loop with water; then the sample is injected onto the column. This is accomplished in 1.05 min. Gradient program and integrator are then started.

(b) *Detectors.*—Waters Model 440 dual wavelength detector (Waters Associates, Inc., Milford, MA 01757) operated at 280 nm with attenuation set at 0.05 AUFS and connected in series with a Hitachi Model 100-10 variable wavelength detector (Thomson Instrument Co., Newark, DE 19711) operated at 230 nm with attenuation set at 0.05 AUFS; or equivalent instrumentation.

(c) *Injector.*—Micromeritics 725 autoinjector equipped with 20 μL loop (Micromeritics Instrument Corp., Norcross, GA 30093), or equivalent.

(d) *3-Pen recorder.*—Soltec Model 3314 (Soltec Corp., Sun Valley, CA 91352), or equivalent.

(e) *Chromatographic column.*—DuPont Zorbax C-8 column, 25 cm × 4.6 mm id (DuPont Instruments, Wilmington, DE 19898), or equivalent.

(f) *Integrator.*—Supergrator 3 (Columbia Scientific Industries Corp., Austin, TX 78766), or equivalent.

(g) *Spectrophotometer.*—Ultraviolet (UV) range. Cary Model 118 (Varian Associates, Inc., Palo Alto, CA 94303), or equivalent.

Reagents

(a) *Water.*—Deionized, distilled, obtained from Milli-Q water purification system (Millipore Corp., Bedford, MA 01730), or equivalent.

(b) *Primary solvent A.*—0.2M NH_4Cl. Weigh 10.7 g NH_4Cl and dissolve in ca 970 mL water. Adjust pH to 3.5 with 10% HCl; add dropwise while the solution is constantly stirred. Measure the pH with pH meter. Transfer solution to 1 L volumetric flask and dilute to volume with water. Prepare fresh daily.

(c) *Secondary solvent B.*—20% (v/v) acetonitrile in methanol. Prepare fresh daily.

(d) *Stock solutions for calibration.*—(1) *Phthalic acid.*—Dissolve 10–14 mg in water in 100 mL volumetric flask and dilute to volume with water. (2) *2-(2′,4′-Dihydroxybenzoyl)benzoic acid.*—Dissolve 8–11 mg in water in 100 mL volumetric flask and dilute to volume with water. (3) *2-(2′,4′-Dihydroxy-3′,5′-diiodobenzoyl)benzoic acid.*—Dissolve 12–14 mg in methanol in 100 mL volumetric flask and dilute to volume with methanol. Determine exact concentration of each stock solution from the UV spectra of aliquots diluted to volume as follows: Dissolve 25 g $(NH_4)_2SO_4$ in ca 60 mL water in a 100 mL volumetric flask; add a 5 mL aliquot of the stock solution and 1 mL HCl and dilute to volume with water. Approximate absorptivities, *a* (L/mg/cm), for these compounds in the same solvent are phthalic acid, 0.045 at 228 nm; 2-(2′,4′-dihydroxybenzoyl)benzoic acid, 0.053 at 286 nm; and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid, 0.056 at 232 nm.

Calibration

Integrator is programmed to integrate peaks on the 230 nm detector for the first 12 min and then to integrate peaks on the 280 nm detector for rest of run.

Construct calibration plots for phthalic acid from integrated area from 230 nm detector and for 2-(2′,4′-dihydroxybenzoyl)benzoic acid and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid from integrated areas from 280 nm detector. Obtain these plots from chromatographic analysis of standard solutions prepared as follows: Dissolve 250 mg FD&C Red No. 3 (shown by previous analysis to be free of intermediates and side reaction products) in water and transfer to a 100 mL volumetric flask. Add appropriate aliquots (0.5–5.0 mL) of stock solutions of the intermediate and 2 side reaction products to the volumetric flask and dilute to volume with water. Analyze 6 calibration solutions containing fairly evenly spaced concentrations of each compound. For each compound in solution, calculate *C*, % by weight relative to the amount of FD&C Red No. 3 in the solution:

$$C = V \times C' \times 100\,(\%) \times (1/250\text{ mg})$$

where V = volume of stock solution aliquot (mL), and C' = concentration of stock solution (mg/mL) determined spectrophotometrically.

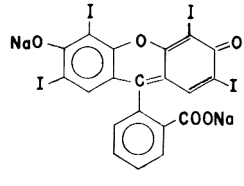

Figure 2. FD&C Red No. 3.

On the basis of current allowable limits, concentrations of phthalic acid, 2-(2′,4′-dihydroxybenzoyl)benzoic acid, and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid, should cover range 0.02–0.2%, expressed as % by weight of FD&C Red No. 3.

Mathematically choose best-fitting straight line for the calibration data by method of least squares. For each intermediate and side reaction product calculate the regression line, $y = bx + a$, by using following equations:

$$b = [\Sigma(x - \bar{x})(y - \bar{y})]/[\Sigma(x - \bar{x})^2]$$

$$= [\Sigma(xy) - [(\Sigma x \Sigma y)/n]]/[\Sigma x^2 - [(\Sigma x)^2/n]]$$

$$a = \bar{y} - b\bar{x}$$

where x = concentration of calibration standard; y = integrator area response to calibration standard for intermediate or side reaction product; n = number of determinations; b = slope of regression line; and a = y intercept of regression line.

Determine linear correlation between peak area and concentration of standards by calculating correlation coefficient r:

$$r = [\Sigma(x - \bar{x})(y - \bar{y})] / \sqrt{[\Sigma(x - \bar{x})^2][\Sigma(y - \bar{y})^2]}$$

Value of r should be between 0.95 and 1.00.

Determination

Dissolve 250 mg FD&C Red No. 3 sample in 30 mL water. Transfer quantitatively to a 100 mL volumetric flask. Dilute to volume with water and mix well. Transfer some of the dye solution to a sample vial. As described under Calibration, prepare a standard FD&C Red No. 3 solution containing each of the 2 side reaction products and the intermediate. Load autosampler in following way: first position, a rinse vial which contains water; second position, a blank gradient vial which contains water; third position, a rinse vial; fourth position, the FD&C Red No. 3 standard; from this position on, all odd-numbered positions contain rinse vials and even-numbered positions contain sample vials. Last sample is the FD&C Red No. 3 standard.

Chromatograph may be started when all samples are loaded in the autoinjector. Place inlet tubes from pumps into the appropriate solvents, which are prepared fresh daily. Pump solvents through lines with column bypass valve opened. Pump long enough to ensure that all lines contain fresh solvents. Set pumps to 20% B at 2 mL/min for 10 min; then set flow to 0 and close bypass valve. Set flow to 1 mL/min and % B to 20; then start injection program. From this point on, chromatograph will operate automatically. After all samples have been run, rinse column with 100% B to remove all buffers; then set flow to 0 and % B to 0; then open bypass valve. Remove inlet tube from primary solvent and place in water. Rinse primary pump with water to remove all buffers from pump and lines. After pump and lines have been rinsed, set flow to 0 and close bypass valve.

From regression line equation, $y = bx + a$, calculate x, the % of each intermediate or side reaction product in the FD&C Red No. 3 sample, by substituting values of y (integrator area for each intermediate or side reaction product) and solving for x.

Resorcinol Detection

If chromatogram of FD&C Red No. 3 sample indicates presence of resorcinol (Figure 3, peak 1), prepare 3 calibration solutions containing 0.05, 0.15, and 0.25 mg resorcinol. For each solution, accurately weigh the resorcinol and 250 mg FD&C Red No. 3 (shown by previous analysis to be free of intermediates and side reaction products), dissolve in water, and quantitatively transfer to a 100 mL volumetric flask. Dilute to volume with water.

Plot peak height vs amount of resorcinol expressed as % by weight of FD&C Red No. 3. These values for resorcinol % by weight should fall within the range 0.02–0.1%. Use calibration plot and peak height of appropriate peak in chromatogram of sample to determine whether resorcinol in sample is under allowable limit.

Results and Discussion

For the development of the method, we used concentrations of the quantitatively determined intermediate and 2 side reaction products in the 9 calibration solutions that covered the following ranges expressed as % by weight of FD&C Red No. 3: phthalic acid, 0.004–0.218%; 2-(2′,4′-dihydroxybenzoyl)benzoic acid, 0.021–0.165%; and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)-benzoic acid, 0.026–0.211%. The 0.004% lower limit of this phthalic acid range is well below the 0.02% lower limit of the calibration range finally suggested in the method, which was designed to meet certification requirements; the recovery studies for all 3 compounds were conducted with amounts added at the 0.02% level or higher. Although the 0.2% upper limit of the calibration range suggested in the method for 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid is the same as the allowable limit, and 0.211% was the highest concentration used in the development

Table 1. Recovery data and paired-difference 99% confidence interval results

	Phthalic acid, %			2-(2′,4′-Dihydroxybenzoyl) benzoic acid, %			2-(2′,4′-Dihydroxy-3′,5′-diiodo-benzoyl)benzoic acid, %		
Detn	Added	Found	Rec.	Added	Found	Rec.	Added	Found	Rec.
1	0.087	0.084	96.6	0.082	0.081	98.8	0.158	0.176	111.4
2	0.065	0.066	101.5	0.124	0.127	102.4	0.211	0.216	102.4
3	0.109	0.108	99.1	0.165	0.165	100.0	0.053	0.052	98.1
4	0.070	0.069	98.6	0.041	0.041	100.0	0.106	0.103	97.2
5	0.052	0.053	101.9	0.062	0.062	100.0	0.132	0.131	99.2
6	0.044	0.044	100.0	0.021	0.021	100.0	0.185	0.205	110.8
7	0.022	0.020	90.9[a]	0.144	0.149	103.5	0.026	0.032	123.1[a]
8	0.087	0.089	102.3	0.103	0.104	101.0	0.079	0.081	102.5
9	0.218	0.210	96.3	0.049	0.049	100.0	0.063	0.066	104.8
10	0.131	0.129	98.5	0.082	0.083	101.2	0.106	0.105	99.1
Av. recovery			99.4			100.7			102.8
Paired-difference 99% confidence interval									
	0.0012 ± 0.0033			−0.0009 ± 0.0018			−0.0048 ± 0.0082		

[a] Point not included in calculations.

of the method, we believed that it was unnecessary to calibrate to a higher level (e.g., 0.3%) because this side reaction product has never been found in a sample of FD&C Red No. 3 submitted for certification.

The following limits of detection were calculated according to the method of statistical analysis described by Bailey et al. (3): phthalic acid, 0.024%; 2-(2′,4′-dihydroxybenzoyl)benzoic acid, 0.011%; and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid, 0.030%.

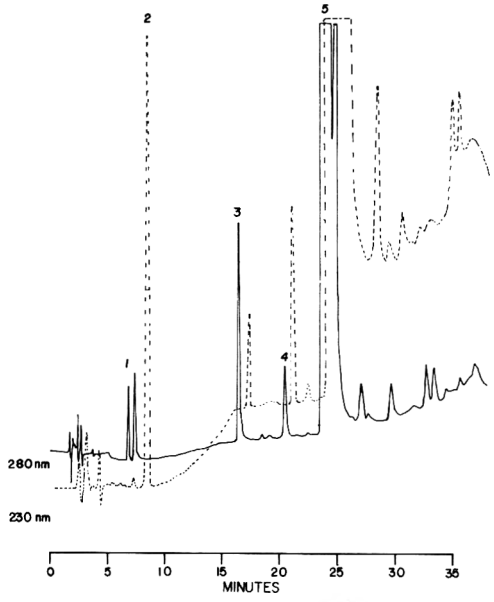

Figure 3. HPLC chromatogram of test solution of FD&C Red No. 3 (peak 5). 1 = resorcinol; 2 = phthalic acid; 3 = 2-(2′,4′-dihydroxybenzoyl)-benzoic acid; 4 = 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid.

Recovery studies with the method gave the following recovery ranges and averages for the intermediate and side reaction products: phthalic acid, 96.3–102.3%, 99.4%; 2-(2′,4′-dihydroxybenzoyl)benzoic acid, 98.8–103.5%, 100.7%; and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)-benzoic acid, 97.2–111.4%, 102.8%. Recovery data for the individual determinations are given in Table 1. The paired-difference 99% confidence intervals all included zero.

A new standard solution should be analyzed every day for the intermediate and for each of the 2 side reaction products. Each set of acceptable calibration data is added to the preceding data and a new regression line and coefficient of correlation are calculated. The correlation coefficient should be between 0.95 and 1.00. A column that produces a correlation coefficient of less than 0.95 should not be used.

Figure 3 shows the separation of resorcinol, phthalic acid, 2-(2′,4′-dihydroxybenzoyl)benzoic acid, and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid. Because C-8 columns vary from company to company, slight modifications in the gradient may be needed to achieve similar results.

Twenty-six commercial samples representing 12 manufacturers were analyzed by the method. Neither side reaction product nor the intermediate phthalic acid was found in any of the samples above the detection limits. No peak was seen in the region where the resorcinol peak (Figure 3, peak 1) or the triiodoresorcinol peak

occurs. (The triiodoresorcinol peak would appear immediately after peak 4, Figure 3, as a shoulder.) These results, which are typical for samples of FD&C Red No. 3 submitted for certification, agree with those obtained for the same samples with the cellulose column/ammonium sulfate eluant procedure (2).

REFERENCES

(1) *Code of Federal Regulations* (1981) Title 21, U.S. Government Printing Office, Washington, DC, sec. 74.303(b)
(2) Link, W. B. (1961) *J. Assoc. Off. Agric. Chem.* **44,** 43–53
(3) Bailey, C. J., Cox, E. A., & Springer, J. A. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1404–1414

ALCOHOLIC BEVERAGES

Determination of Proof by Distillation: Low Results for Cocktails and Specialty Products

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A variety of specialty products, as well as alcohol solutions containing individual ingredients of the products, were proofed according to the accepted distillation method for beverages containing ≥600 mg extract/100 mL. Products and solutions containing non-dairy creamer, combinations of citric acid and sugar, or acetic acid gave distillates with specific gravities corresponding to proofs 0.2-0.7° lower than expected. Low results caused by carryover of acetic acid into the distillate can be prevented by making the sample alkaline before distillation. The mechanism of apparent reduced recovery of alcohol in the other cases is still under investigation.

A search for the causes of alcohol losses during the manufacture of cocktails and specialty products has produced evidence that the losses do not occur during processing and handling. Attention has, therefore, turned to the methods and instrumentation used to measure proof.

Because the products of interest contain large amounts of solids, the normal method of determining proof requires distillation of the sample in a laboratory still followed by measurement of proof of the distillate by hydrometer or pycnometer. Improved accuracy, obtained by using pycnometers and the density meter (1, 2), has confirmed that some losses can be attributed to erroneously low proof results on products in the lower proof range (≤60° proof).

The present study is an investigation of the laboratory distillation procedure. It is known that it is theoretically impossible to recover all the alcohol in a distillation pot. The amount left in the pot is a function of condenser efficiency, distillation rate, and the composition of the pot. This study will demonstrate the importance of the composition of the pot in the distillation and recovery of alcohol from complex products.

Experimental

Alcohol–water solutions and selected cordial and specialty products having known proofs were prepared by the following method: The calculated amount of alcohol was weighed into a volumetric flask, water or other ingredients were added as required, and the solution was diluted almost to volume. The flask was stoppered and cooled to 60°F, and final dilution to volume was made with water cooled to 60°F.

Distillations were carried out in a simple still, using a 500 mL distillation pot connected to a vertical condenser, as described in the official method (3). A 100 mL sample, diluted in the pot with 100 mL water, was distilled in 30–35 min; 96 mL distillate was collected, adjusted to the temperature of the original sample, and diluted to volume with distilled water. (The pot residue from certain products will scorch before distillation is complete if only 40 mL rinse is used, as specified in the official method.)

Proofs of all solutions and distillates were determined by density meter.

Results and Discussion

Table 1 shows proof results obtained after distillation of various alcohol–water solutions. Comparison of actual proof, determined before distillation, with calculated proof demonstrates the accuracy with which solutions can be prepared by the method described above. The difference between actual proof and distillate proof shows that even in this simple system, alcohol–water, proof determined after distillation is approximately 0.1° proof low.

Table 2 shows results obtained with a representative selection of cordial and specialty products. Calculated proofs are assumed to be as accurate as those obtained with the alcohol–water system. In all cases the distillate proof is lower than the calculated proof. For example, for creme de cacao and anisette, the difference is approximately the same as for alcohol–water solutions of the same proof. For the other products, representing various specialties, the distillate proof ranges from 0.2 to 0.7° proof less than the calculated proof.

Table 3 shows results obtained with bloody mary product and with alcohol solutions of various individual ingredients of bloody mary product. The concentration of each individual

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Table 1. Proof loss in distillation method for various distilled spirits

Product	Calcd proof	Actual proof	Distillate proof[a]	Diff.
Spirits	54.00	54.00	53.84	−0.16
Spirits	40.00	40.04	39.88	−0.16
Vodka	35.00	34.99	34.85	−0.14
Rum	30.00	29.98	29.87	−0.11
Rum	25.00	24.97	24.88	−0.09
Tequila	25.00	25.01	24.94	−0.07
Vodka	20.00	20.00	19.90	−0.10

[a] All values represent the average of 4–8 replicate determinations.

Table 2. Comparison of calculated proof with distillation proof of cordials and specialty products

Product	Calcd proof	Distillate proof[a]	Diff.
Creme de cacao	54.00	53.89	−0.11
Anisette	40.00	39.84	−0.16
Gimlet	35.00	34.80	−0.20
Daiquiri	30.00	29.74	−0.26
Pina colada	25.00	24.66	−0.34
Tequila sunrise	25.00	24.79	−0.21
Bloody mary	20.00	19.30	−0.70

[a] All values represent the average of 4–8 replicate determinations.

Table 3. Comparison of calculated proof with distillation proof of bloody mary and ingredients

Sample	Calcd proof	Distillate proof[a]	Diff.
Vodka & tomato juice	20.0	19.9	−0.1
Vodka & seasonings	20.0	19.9	−0.1
Vodka & vinegar	20.0	19.4	−0.6
Bloody mary	20.0	19.3	−0.7
Bloody mary made alk. before distn	20.0	19.9	−0.1

[a] All values represent the average of 4–8 replicate determinations.

Table 4. Comparison of calculated proof with distillation proof of pina colada

Non-dairy creamer, % usage level	Calcd proof	Distillate proof[a]	Diff.
0	25.00	24.87	−0.13
50	25.00	24.72	−0.28
100	25.00	24.66	−0.34
120	25.00	24.62	−0.38

[a] All values represent the average of 4–8 replicate determinations.

ingredient was the same as its usage level in the product. The solution of vodka and vinegar shows a low result almost equal to that seen with the bloody mary product. Solutions of the other ingredients show only the same low result (0.1° proof) seen with alcohol–water solutions of the same proof. In this case, the apparent reduced recovery of alcohol can be attributed to an increase in specific gravity of the distillate caused by carry-over of acetic acid. It was therefore necessary to make the solution in the distillation pot alkaline by adding, before distillation, sodium hydroxide in excess of the amount needed to neutralize the acetic acid. The last item in Table 3 shows the proof result obtained after distillation of an alkaline bloody mary sample. This result is also 0.1° proof lower than the calculated proof.

Table 4 shows results obtained with a series of pina coladas containing various levels of non-dairy creamer. Low results obtained after distillation range from 0.1° proof low, which has occurred with all distillations, to 0.34° proof low for the commercial product (represented by 100% usage level non-dairy creamer), and 0.38° proof low when an excess of non-dairy creamer is used. The trend is clear and shows the low proof results to be a function of the non-dairy creamer concentration. The mechanism is unknown. The possibility of an interaction between alcohol and the casein component of the non-dairy creamer is being investigated. Likewise, neither mechanism nor cure has been found for the low results obtained with the other specialty products shown in Table 2.

It appears possible, therefore, to measure alcohol into a product or solution in a precise way and yet be unable to demonstrate the presence of that alcohol by the accepted method of proof determination. Studies will continue in an attempt to find the cause for the low results and to find a method for determining alcohol content accurately. Results of this initial work are presented to demonstrate the difficulties of determining alcohol content in complex products and to call attention to the persistent 0.1° proof loss which occurs in all distillations.

References

(1) Strunk, D. H., Hamman, J. W., & Timmel, B. M. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 653–658.
(2) Mark, F. G., & Vaughn, T. E. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 970–972
(3) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs 9.012–9.013

PESTICIDE FORMULATIONS

Gas-Liquid Chromatographic Method for Analysis of Pentachloronitrobenzene Formulations: Collaborative Study

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A collaborative study has been conducted on a gas-liquid chromatographic (GLC) method for determining pentachloronitrobenzene (PCNB) in formulations. Wettable powder, liquid, and granular matched pairs of commercial products were analyzed by 17 laboratories using peak height measurements and by 12 laboratories using integrator area measurements. Samples were dissolved in chloroform and aliquots were mixed with internal standard before GLC analysis on a 5% SE-30 column. Mean coefficients of variation for the completed study were 1.54% for integrator area measurements and 1.35% for peak height measurements. The method has been adopted official first action.

A gas-liquid chromatographic method for the determination of the fungicide pentachloronitrobenzene (PCNB, Terrachlor, Quintozene) in fertilizers was first reported in 1972 (1). The column packing and internal standard were changed and the modified method was applied to dust, powder, liquid, granular, and fertilizer formulations containing PCNB (2). The latter method was subjected to collaborative study.

Each collaborator was furnished with 6 samples (3 matched pairs) and asked to perform a single analysis on each sample. Included with the samples were the published method (2), PCNB analytical standard, the internal standard (*o*-terphenyl), 5% SE-30 column packing, and report forms. Collaborators were instructed to use peak height measurements for PCNB calculations and integrator area measurements if this method was commonly used in their laboratories. In addition, it was requested that each collaborator return all data and chromatograms.

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This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and Committee A and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* **65**, March issue (1982) for detailed reports.

Pentachloronitrobenzene (PCNB) — Gas-Liquid Chromatographic Method

Official First Action

Principle

Sample is dissolved in $CHCl_3$, *o*-terphenyl is added as internal std, and PCNB is detd by GLC with flame ionization detection.

Apparatus and Reagents

(a) *Gas chromatograph with recorder.*—With flame ionization detector and 1.8 m × 4 mm (id) glass column packed with 5% SE-30 on 80–100 mesh Chromosorb W (dimethylchlorosilane-treated) (Analabs, Inc.). Condition newly packed column 24 h at 285° with low N flow. Operating conditions: temps (°)—inlet 200, column 175–180, detector 250; carrier gas flow to elute PCNB at ca 4.5 min; adjust H and air as recommended for detector by manufacturer; sensitivity to give peak heights 60–80% full scale.

(b) *PCNB std soln.*—2.0 mg/mL $CHCl_3$. Accurately weigh ca 0.2 g PCNB (Olin Corp., Agriculture Products Dept, PO Box 991, Little Rock, AR 72203) into 100 mL vol. flask and dil. to vol. with $CHCl_3$.

(c) *Internal std soln.*—0.8 mg/mL $CHCl_3$. Accurately weigh ca 0.2 g *o*-terphenyl (Eastman Kodak Co.) into 250 mL vol. flask and dil. to vol. with $CHCl_3$. Discard after 3 days.

(d) *Mixed std soln.*—1.0 mg PCNB + 0.4 mg *o*-terphenyl/mL. Pipet 25 mL each of PCNB and internal std solns into vial and mix.

Preparation of Sample

(a) *Solid formulations.*—Grind 100 g well mixed sample to pass 1 mm sieve. Accurately weigh portion of well mixed, ground sample contg ca 0.2 g PCNB into 250 mL g-s erlenmeyer and add

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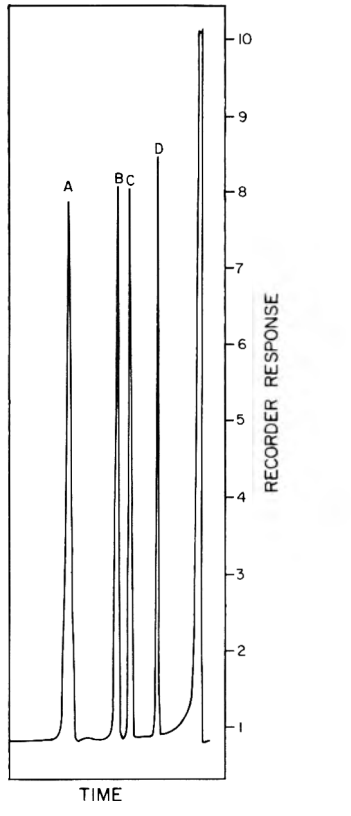

Figure 1. Gas chromatogram of mixed standard and PCNB impurities: A, o-terphenyl; B, PCNB; C, hexachlorobenzene; D, tetrachloronitrobenzene.

100 mL $CHCl_3$; stopper and shake 2 h on rotary shaker. Let insoluble matter settle.

(b) *Wettable powders.*—Accurately weigh portion of well mixed sample contg ca 0.2 g PCNB into 250 mL g-s erlenmeyer and proceed as for solids.

(c) *Liquids.*—Accurately weigh portion of well mixed sample contg ca 0.2 g PCNB into 100 mL vol. flask and dil. to vol. with $CHCl_3$.

(d) *Soln for analysis.*—Pipet 10 mL sample ext above and 10 mL internal std soln into vial, cap, and mix.

Determination and Calculation

Inject 4 μL aliquots of mixed std soln until variation in response ratio (area or peak height) for PCNB (first peak) to *o*-terphenyl (second peak) is ca 1%. Inject mixed std, inject sample twice, and repeat injection of mixed std. Retention times must be the same for sample and std. Calc. av. ratios of PCNB to *o*-terphenyl for the 2 mixed std and sample injections, and calc. % PCNB.

$$\% \text{ PCNB} = (R/R') \times (W'/W) \times P$$

Where R and R' = av. response ratios for sample and mixed std, resp.; W' = g PCNB/100 mL std soln; W = g sample extd; and P = purity (%) of PCNB std.

Discussion

In the analysis of any pesticide formulation it is necessary to consider potential interferences from impurities. The primary impurities in PCNB are hexachlorobenzene (HCB), tetrachloronitrobenzene (TCNB), and pentachlorobenzene (PCB).

Figure 1 illustrates the chromatography of 2 of these compounds, HCB and TCNB, along with PCNB and the internal standard (*o*-terphenyl). PCB, which is not included in the chromatogram, would elute before TCNB. The only potentially interfering impurity, HCB, is resolved from PCNB, even at approximately equal concentrations (Figure 1). The usual level of HCB is less than 0.5% of PCNB. In some countries there are specific legal constraints on the levels of HCB and PCB. Determining these impurities while determining PCNB by the method used in this study has not been tested; however, the problem of simultaneous determination of PCNB plus impurities is being addressed by the Dutch Pesticides Analytical Committee (A. Martijn, Plantenziektenkundige Dienst, 1980).

Collaborative Study

The sample pairs analyzed in the collaborative study were selected to represent the broad spectrum of formulated products containing PCNB as follows: for powders and dusts, 75% wettable powders; for liquids, 25% emulsifiable concentrates; and for granules and fertilizers, 10% granules. The study was designed to follow Youden's procedure for closely matched pairs (3). All samples were collected during routine pesticide inspections.

Table 1 gives the collaborative results for all determinations made using peak height measurements, while the results obtained for integrator area measurements are given in Table 2. Matched pair calculations were performed according to Youden's method. Confidence intervals of 99% (2-tail t values at the probability of 0.01) were applied to the sums of paired results to identify outlier laboratories. None was identified. Similarly, application of 99% confidence intervals to differences between paired results identified outliers as indicated in Tables 1 and 2. Two-sample plots were constructed to

Table 1. Collaborative results for PCNB by peak height measurements

	Pair 1		Pair 2		Pair 3	
Coll.	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
1	75.02	73.75	25.95	25.11	10.29	10.47
2	74.62	73.99	26.00	25.35	10.16	10.52
3	74.11	73.15	26.22	25.06	9.97	10.61
4	74.60	72.34	25.83	25.15	10.48	10.70
5	75.00	73.76	26.32	25.69	10.23	10.65
6	75.20	73.64	25.15[a]	25.51[a]	10.05	10.59
7	78.05	76.06	26.28	25.04	10.77	10.78
8	75.77	73.80	26.37	24.96	10.29	10.70
9	74.48	74.09	25.97	25.23	9.90	10.55
10	74.58	73.36	26.56	25.59	10.16	10.82
11	75.18	73.73	26.21	25.53	10.14	10.67
12	74.74	73.64	26.13	25.29	10.03	10.69
13	74.62	73.48	26.03	25.22	10.07	10.57
14	77.60[a]	74.06[a]	26.31	25.84	10.21	10.66
15	75.68	74.47	26.59	25.77	10.32	10.75
16	74.65	73.81	25.88	24.27	10.02[a]	9.58[a]
17	73.44	72.77	25.76	25.15	10.09	10.52
Mean	74.98	73.74	26.15	25.27	10.20	10.64
S_d (CV)	0.90 (1.21)		0.32 (1.25)		0.16 (1.53)	
S_r	0.36		0.22		0.13	
S_b	0.82		0.23		0.10	

[a] Outlier by pair difference.

visually confirm the statistical rejections.

After rejection of outliers, the random, actual data, and bias standard deviations (S_r, S_d, and S_b) were determined for each pair for both methods of measurements (Tables 1 and 2). Youden *F*-test ratios (3) were greater than the 99% critical values for Sample pair 1 by both peak height and integrator area measurements, indicating the presence of bias or systematic error. Further comparison of the results is given in Table 3. The average precision is slightly better for peak height results. Visual comparison of the sample means indicates potential differences in the results obtained by the 2 methods of measurement for Sample pair 1. *t*-Test calculations on Sample pair 1 differences of pair totals obtained by the 2 methods gives a *t* value considerably less than the 99% critical value. Thus, no statistical dif-

Table 2. Collaborative results for PCNB by integrator area

	Pair 1		Pair 2		Pair 3	
Coll.	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
1	75.12	73.79	26.11	25.48	10.21	10.76
2	75.21	74.14	26.01	25.34	10.85	10.52
4	76.33	75.78	26.18	25.92	10.39	10.88
6	75.27	73.67	25.12	25.50	10.04	10.60
8	76.20	74.20	26.07	24.99	10.18	10.68
10	75.04	73.59	26.70	25.51	9.95	10.81
11	75.09	73.94	26.23	25.38	10.14	10.64
12	75.04	73.61	26.09	25.29	9.99	10.61
13	74.66	73.44	25.97	25.22	10.09	10.53
14	76.52	74.63	26.40	25.71	10.36	10.83
15	75.82	74.43	26.58	25.75	10.27	10.74
16	76.00	74.36	27.40	24.81	10.00[a]	9.58[a]
Mean	75.52	74.13	26.24	25.41	10.21	10.60
S_d (CV)	0.63 (0.84)		0.44 (1.70)		0.197 (1.89)	
S_r	0.27		0.48		0.204	
S_b	0.56		—[b]		—[b]	

[a] Outlier by pair difference.
[b] $S_r > S_d$ by chance; S_b obscured.

Table 3. Peak height and integrator area calculations of PCNB

	PCNB, av. (%)		CV of pairs	
Sample	Peak ht	Integr. area	Peak ht	Integr. area
A	74.98	75.52	1.21	0.84
B	73.74	74.13		
C	26.15	26.24	1.25	1.70
D	25.27	25.41		
E	10.20	10.21	1.58	1.89
F	10.64	10.60		
Av.			1.35	1.54

ference is indicated in the sets of results obtained by peak height and integrator area measurements; the Associate Referee recommends that the method of peak measurement be left to the discretion of the analyst.

Collaborators generally found the method easy to use and encountered few problems. Collaborator 1 reported that the internal standard solution did not appear stable for more than 3 days. Instructions to discard the internal standard solution after 3 days have been added to the method. The Associate Referee will further investigate internal standard stability. Collaborator 2 used a magnetic stirrer in place of a rotary shaker, while Collaborator 10 suggested that laboratories without a rotary shaker might use a wrist-action shaker with resultant loss of solvent due to splash onto the glass stopper. A screw-cap flask could be used under these circumstances. Collaborator 4 repeated the analysis of samples with a column packed with 10% SP-2401 and obtained comparable results with a shorter analysis time and reversal of the elution order for PCNB and *o*-terphenyl. The Associate Referee does not recommend the use of alternative column packings without complete evaluation of potential interferences. In addition to evaluating results for peak heights with the internal standard, Collaborator 13 also determined PCNB in the samples by ignoring the internal standard, i.e., using external standard calculations. An auto-injector was used and the results were essentially the same with or without the internal standard. It appears that the injection precision of auto-injection compensates for the precision lost by ignoring the internal standard. The Associate Referee recognizes the value of screening samples without using an internal standard. Finally, Collaborator 16 reported that the peak heights for both PCNB and internal standard were unusually low for Sample pair 2. The levels of PCNB found in the samples were also low. After he adjusted the hydrogen more in line with the recommended flow for his GLC system, both peak heights and results of analysis increased. The Associate Refereee examined the chromatograms from all other collaborators and did not find a decrease in peak heights for Sample pair 2 to be a problem. The make and model of GLC apparatus used by Collaborator 16 was the same as that used by other collaborators. The results of Collaborator 16 on Sample pair 2 were retained for statistical analysis and were not rejected as outliers.

Although one basic principle of a collaborative study is the use of the same conditions by each collaborator, this is seldom achieved and differences in instrumentation may be a source of variation. The operational parameters used for the analysis of PCNB by the collaborators in this study are given in Table 4. Column temperature is the primary parameter that was altered. Comments from some of the collaborators indicated a higher temperature than specified was needed to achieve the recommended retention time for PCNB. The source of this problem is probably 2-fold: differences in column packing techniques used by the collaborators and differences in the upper limit of carrier gas flow that can be used with detectors of various manufacture. In spite of use of elevated column temperatures by some of the collaborators, none of the outlier results is identified with this method parameter alteration. Only changes in the recommended column diameter and the injection volume are associated with outliers. Since other collaborators used similar condition alterations, it appears that the outliers can not necessarily be attributed to these parameters. Thus, the changes in method conditions used by the collaborators probably did not affect the results, and the alterations used tend to complement the previously demonstrated ruggedness of the method (2).

Recommendations

Ninety-nine percent confidence limits were used to evaluate the data from the collaborative study to retain the maximum number of results. This approach gives a more accurate picture of method performance than that provided by over-censoring the data with narrower limits. As a result, the precision of the method may be slightly poorer than expected of a GLC method and an internal standard; however, the predicted method performance is a realistic reflection of the variety of conditions likely to be encountered in different laboratories. Thus, the Associate

Table 4. Collaborator method parameters

Coll.	Injection vol., μL	Column, mm		Temperature, °C			Carrier gas[a]
		id	Length	Inlet	Column	Detector	
1	6	4	1828	200	180	250	H
2	4.5	4	1828	200	200	250	N
3	4	4	1524	200	175	245	N
4	4	4	1828	200	175	250	H
5	4	—	1828	—	180	300	N
6[b]	3	2	1828	200	175	250	N
7	4	4	1828	—	175	250	N
8	5	4	1828	200	175	250	N
9	6	4	1828	175	175	250	N
10	3.5	4	1828	200	175	250	N
11	4	4	1828	200	190	250	N
12	4	4	1828	220	175	250	N
13	4	4	1828	200	190	250	N
14[b]	4	—	1828	200	175	250	N
15	4	2	1828	200	175	250	N
16[b]	4	2	1828	200	175	250	N
17	4	2	1828	200	170	250	N
Specified cond.	4	4	1828	200	175	250	N

[a] H = helium; N = nitrogen.
[b] Collaborator with outlier results.

Referee recommends that the GLC method collaboratively studied for determination of PCNB in formulations be adopted official first action.

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References

(1) Hanks, A. R., & Engdahl, B. S. (1972) *J. Assoc. Off. Anal. Chem.* **55**, 657–659

(2) Hanks, A. R., & Cramer, C. W. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 708–710

(3) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

Gas-Liquid Chromatographic Method for the Analysis of Microencapsulated Diazinon Insecticide: Collaborative Study

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The determination of diazinon insecticide in Knox Out 2FM formulation was studied collaboratively by 18 laboratories. Knox Out 2FM is a flowable microencapsulated insecticide formulation containing 23 wt% active ingredient. Analytical samples are first treated by grinding in a tissue grinder and then extracted in situ with acetonitrile. This preparative step breaks the capsules and allows the active ingredient to dissolve in the solvent. Single determinations on each of 2 closely matched samples were made by flame ionization gas-liquid chromatography. The standard deviation by analysts was 0.18 wt% and the coefficient of variation was 0.76%. The combined laboratory and analyst variation gave a standard deviation of 0.59 wt% and a coefficient of variation of 2.49%. The method has been adopted official first action.

Knox Out® 2FM is a flowable microencapsulated formulation containing 2 lb diazinon/gal. Its controlled release allows excellent residual kill of cockroaches and silverfish. The formulation is used for residual pest control in and around residential buildings including homes and apartments, and in and around non-food areas of industrial, institutional, and commercial buildings, including but not limited to stores, hospitals, manufacturing plants, warehouses, and schools.

The reported acute oral LD_{50} (rat) for microencapsulated diazinon (Knox Out 2FM) is greater than 21 000 mg/kg, making it one of the safest insecticidal formulations marketed. Knox Out 2FM is unique in that it consists of an insecticide contained within small, bubble-like polymeric capsules about 30–50 μm in diameter. The sustained release of the active ingredient is controlled by the thickness of the capsule walls, which varies according to the size of droplets they contain. Diazinon is retained or released through the wall, depending on the amount of surrounding moisture. Water tends to keep the insoluble active ingredient in the capsule; pesticide is released when water around the capsule evaporates after application.

Gas-liquid chromatography (GLC) is the preferable analytical procedure, because of its rapidity, specificity, and reliability. Several years of continued research on this methodology have established confidence in the method's applicability. The method specifies the most suitable sampling technique along with proper sample preparation which allows for quantitative determination by flame ionization GLC (Figure 1).

Collaborative Study

The analytical procedure was evaluated by having several laboratories systematically analyze production batches of Knox Out 2FM. According to Youden, it is best to select 2 closely matched samples and make one determination on each; however, these 2 samples must be quite close in content and very similar in composition. Each collaborator received a matched pair of samples labeled A and B, representing production batches with diazinon content approximately 23 wt%. Each collaborator also received a glass tissue grinder as needed, internal standard, analytical grade diazinon, column packing, weighing dishes, safety data sheet, and a copy of the method. Each laboratory was requested to analyze each sample only once and to report individual values obtained for Samples A and B. For those laboratories which had never used a tissue grinder, it was advised that a practice sample be prepared before preparing the analytical sample. Mistakes in technique could be corrected at that time.

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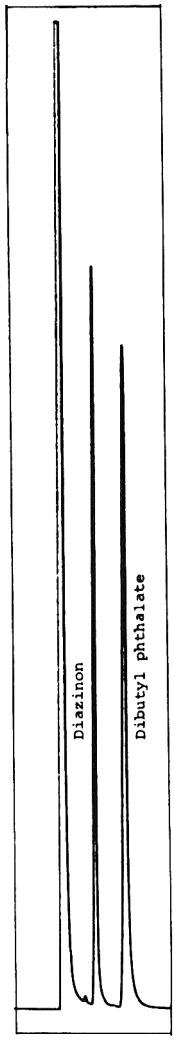

Figure 1. Chromatogram for 1 μL injection of standard solution of 0.2 g each diazinon and dibutyl phthalate/50 mL acetonitrile.

Microencapsulated Diazinon – Gas-Liquid Chromatographic Method

Official First Action

Principle

Sample is ground in tissue grinder and extd with acetonitrile, dibutyl phthalate is added as internal std, and diazinon is detd by GLC with flame ionization detection.

Apparatus

(a) *Gas-liquid chromatograph.*—Equipped with flame ionization detector (Perkin-Elmer 900, or equiv.).

(b) *GLC column.*—6 ft × 1/4 in. od, 2 mm id glass, packed with 3% OV-17 on 80–100 mesh Supelcoport. Operating conditions: column 190° (isothermal); detector 250°; injection port 200°; He flow 35 mL/min; H flow optimum for instrument detector chart speed 0.2 in./min; sample size; 1 μL.

(c) *Tissue grinder.*—40 mL capacity (Corning Glass Works, No. 441969, Corning, NY 14830), or equiv.

Reagents

(a) *Internal std soln.*—Accurately weigh ca 2.0 g dibutyl phthalate into 100 mL vol. flask. Dissolve in and dil. to vol. with acetonitrile.

(b) *Diazinon standard solution.*—Accurately weigh ca 0.2 g diazinon into 50 mL vol. flask. Pipet in 10.0 mL internal std soln, dil. to vol. with acetonitrile, and mix well.

Determination

Mix sample thoroly. With medicine dropper or disposable pipet, transfer ca 2 g sample to Al weighing dish and weigh accurately. Transfer to tissue grinder, add 30 mL acetonitrile, and grind 3 min. When sample is thoroly ground, quant. transfer to 100 mL vol. flask, wash grinder with acetonitrile, and add washings to vol. flask. Pipet in 20.0 mL internal std soln and dil. to vol. with acetonitrile. Using 10 μL syringe, make duplicate 1 μL injections of sample and std solns.

Measure peak heights of first peak, diazinon, in sample (PH) and std soln (PH') and second peak, dibutyl phthalate, in sample (IS) and std soln (IS').

$$\text{Diazinon, wt\%} = (PH/PH') \times (IS'/IS) \times (W'/W) \times P$$

where W and W' = g diazinon and internal std in std soln; and P = purity of diazinon std.

Results and Discussion

Statistical Evaluation

Collaborative results are given in Table 1. The statistical study is based on single determinations on each of a matched pair of samples by 18 collaborators. An initial review of the data would indicate results from Collaborators 6 and 11 to be gross outliers. A 2-sample plot was constructed (Figure 2) which shows that those results and results from Collaborator 5 are outliers. Systematic errors for the laboratories supplying these data are evidentally large in comparison with the other collaborators. Outliers which are

Table 1. Collaborative results for determination of diazinon in encapsulated formulations

Coll.	Sample A, wt%	Sample B, wt%	Diff.	Total
1	23.68	22.97	0.71	46.65
2	24.00	23.40	0.60	47.40
3	24.05	23.86	0.19	47.91
4	24.17	23.96	0.21	48.13
5[a]	24.80	23.30	1.50	48.10
6[a]	19.46	19.37	0.09	38.83
7	24.30	24.00	0.30	48.30
8	23.67	22.89	0.78	46.56
9	22.47	22.06	0.41	44.53
10	24.30	23.80	0.50	48.10
11[a]	26.50	26.32	0.18	52.82
12	25.17	24.26	0.91	49.43
13	23.94	23.65	0.29	47.59
14	24.28	24.13	0.15	48.51
15	23.36	22.95	0.41	46.31
16	24.02	23.80	0.22	47.82
17	23.68	22.97	0.71	46.65
18	23.90	23.77	0.13	47.67

[a] Outlier laboratory results omitted from calculations.

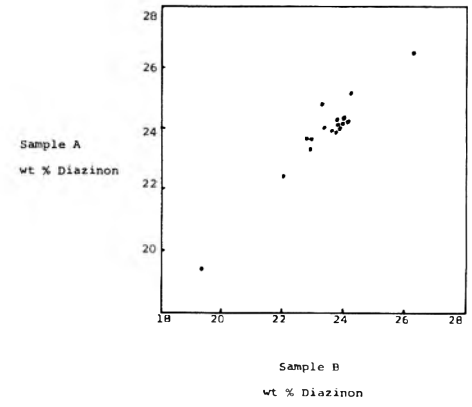

Figure 2. Two-sample plot of collaborative sample results.

identified in Table 1 exceed the 98% confidence limits; only the results from the remaining 15 collaborators were used to develop the standard deviation and coefficient of variation data.

The standard deviation of analysts (S_r) was 0.18 wt%, while the standard deviation for laboratories (S_b) was 0.56 wt%; the combined variation (analysts plus laboratories) resulted in a standard deviation of 0.59 wt% (Table 2). The corresponding data for the coefficient of variation for analysts was 0.76%, and for laboratories, 2.38%. The coefficient of variation (combined) was 2.49%.

Collaborators' Comments

Collaborator 16 recommended pipeting a known amount of internal standard into the sample and standard solutions, which would eliminate transfer errors. This was accepted. Also, Collaborator 16 used an Omni-Mixer (DuPont) blender with a 50 mL stainless steel cup for sample preparation. His results for a single analysis on Samples A and B agree with the data in Table 1.

Collaborator 13 used a shorter analytical column than the one recommended in the method. Nevertheless, by optimizing column and operating parameters, he was able to obtain satisfactory data.

Collaborator 14 proposed using sharp sand as a grinding aid in the sample preparation step: "Prewash sharp sand with acetone and dry. Place enough sand in a 125 mL Erlenmeyer flask to cover flask bottom. Weigh sample into flask directly on top of sand and extract 4 h with acetonitrile containing internal standard." His analytical data agree with the collaborators' results. This author does not disagree with this practice; however, in the event of questionable data, the tissue grinder technique is preferred. This is the only sample preparation technique that has been subjected to the validation process.

Collaborator 12 substituted his own analytical column with a Tracor Model 222 gas chromatograph and Hewlett-Packard 3380 A recording integrator. Results were expressed as electronic peak area measurements and were consistent with the results of the study.

Collaborator 11 experimented with the use of a kaolin clay as the grinding medium in lieu of using the tissue grinder: "Weigh 2 g samples into 250 mL Erlenmeyer flask, and add 20 g sand, 20 mL internal standard, and 80 mL acetonitrile. Mechanically shake samples 2 h, let settle, and inject 1 μL into GLC system." These variations involve less sample handling and no special glassware. Using sea sand under similar conditions, he found 23.55% and 25.54% diazinon. Using 16–40 mesh Little Rock kaolin clay, he

Table 2. Statistical analysis of data in Table 1

Statistic	SD	CV, %
Analyst variation only, S_r	0.18	0.76
Laboratory variation only, S_b	0.56	2.38
Combined variation $\sqrt{S_r^2 + S_b^2}$	0.59	2.49

found 27.12% and 26.79%, respectively. Kaolin clay causes the sample to break into small pieces and mix in the grinding medium, while sea sand permits the sample to remain as a thick film on the bottom of the flask. Unfortunately, the systematic error for the laboratory supplying data for this technique is large in comparison with the other collaborators.

Collaborator 7 also suggested the alternative use of sharp sea sand which is routinely used in state laboratories to analyze encapsulated products. "Addition of 2–3 g sharp sand (washed and ignited) to the sample in a 125 mL Erlenmeyer flask followed by 1 h of shaking is all that is necessary for sample preparation. The method is effective, rapid, and clean (no transfer necessary). Our analyses of samples A and B by this method resulted in 22.6% and 22.5% diazinon, respectively." Collaborative study data developed by Collaborator 7 were consistent with the data in Table 1; however, the analytical data points obtained for the sharp sand technique qualify as acceptable, but only marginally.

Collaborator 6 submitted chromatograms along with the analytical data. An initial analysis gave low values. A second analysis with a new gas chromatographic column provided higher analytical results; however, inspection of the chromatograms casts doubt on the acceptability of these data. A 2-sample plot of the results clearly showed the data to be outliers.

Recommendation

The results of the collaborative study indicate excellent agreement among laboratories along with high precision among analysts and satisfactory ruggedness. Invariably, collaborators introduce modifications in the procedure to satisfy their analytical judgment. The myriad changes introduced by several collaborators in the sample preparation step showed no evidence of data degeneration. Also, use of area measurements in place of peak height measurements along with column substitution and variations in sample size did not introduce inconsistencies in the results. It is therefore recommended that the gas-liquid chromatographic method for the determination of diazinon in encapsulated formulations be adopted official first action.

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N. D. Ellis and A. K. Torrence, College of Agricultural Sciences, Clemson, SC

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R. D. Shah, Union Carbide Agricultural Products Co., Ambler, PA

L. Ullrich, Colorado Dept of Agriculture, Denver, CO

R. L. Whitaker, Pennwalt Corp., Bryan, TX

G. R. Winstead III, and D. L. Wong, Dept of Agriculture, Raleigh, NC

FISH AND OTHER MARINE PRODUCTS

Fish Species Identification by Thin Layer Agarose Isoelectric Focusing and Densitometric Scanning

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Examination of sarcoplasmic proteins of fish by thin layer polyacrylamide gel isoelectric focusing as a means of fish species identification is a powerful and reliable technique, but it displays a number of disadvantages. These problems include the care required when handling acrylamide monomer (a neurotoxin), the mechanical skill needed in molding the gel, the difficulties in ensuring correct gel polymerization, and the extensive destaining periods. Specially treated agarose has been used to obtain protein patterns for an interval of pH 5-8. The patterns so produced were scanned by a densitometer in the visible range after completing staining and destaining. Most of the problems associated with polyacrylamide gels have been overcome by using agarose as a support medium.

Agarose has been used for many years in electrophoresis, particularly immunoelectrophoresis (1), but agarose is unsuitable for electrofocusing because of the electroosmosis effect caused by interaction of the high field strengths and low conductivities experienced in electrofocusing with the residual charge on the agarose matrix (2). During an experiment this effect leads to solvent drift to one of the electrodes, commonly the cathode. This results in areas of flooding and drying, causing severe disruption of pH gradient and mechanical stability of the gel.

The low residual charge in electrophoresis grade acrylamide, as opposed to the relatively high residual charge in agarose, has led to the widespread use of polyacrylamide gels for electrofocusing. A new form of agarose has, by and large, overcome the limitations of agarose as a support medium in isoelectric focusing by lowering the residual charge by chemical means.

Isoelectric focusing has been successfully applied to the identification of fish species (3–6) for both wide (pH 3.5–10.0) and narrow range (pH 4–6 and pH 3.5–7) gradients. All experiments in the quoted references were carried out with polyacrylamide as support medium. Although results obtained from polyacrylamide isoelectric focusing are specific and reproducible, polyacrylamide suffers from a number of drawbacks.

The use of acrylamide requires extreme care because of toxicity and because of the difficulties sometimes experienced in gel polymerization. Polymerization occurs only with the use of high purity chemicals, fresh solutions, proper degassing periods, and adequate time for correct polymerization. It is by no means a rare event to encounter a gel of poor mechanical stability even when it appears that all criteria have been met.

Polyacrylamide gels require extensive destaining periods and need delicate handling, especially when the gel separates from the glass backing plate. Tears are a common occurrence when photographing or scanning by densitometer. The difficulties experienced can lead to poor results and affect the acceptance of isoelectric focusing as a technique. Of course, the difficulties arising from laboratory casting of polyacrylamide plates may be overcome by the use of factory-prepared polyacrylamide plates but the problems arising from toxicity and destaining periods still remain.

Chemically treated agarose (agarose IEF) offers certain advantages regarding handling, safety, time, and convenience compared with polyacrylamide. Fish samples were subjected to isoelectric focusing on agarose as a support medium within a pH gradient of 5–8. This range was chosen to take advantage of the fact that agarose IEF is formulated for minimal cathodic drift between pH 5 and pH 8. Because the characteristic sarcoplasmic protein bands fall largely in this region, the protein patterns obtained after staining are simpler and generally more widely spaced than those given with a wide range pH 3–10 gradient.

METHOD

Reagents

(a) *Agarose.*—Agarose IEF (Pharmacia Fine Chemicals AB, Sweden).

(b) *Sorbitol.*—AR grade (BDH Chemicals Ltd, UK).

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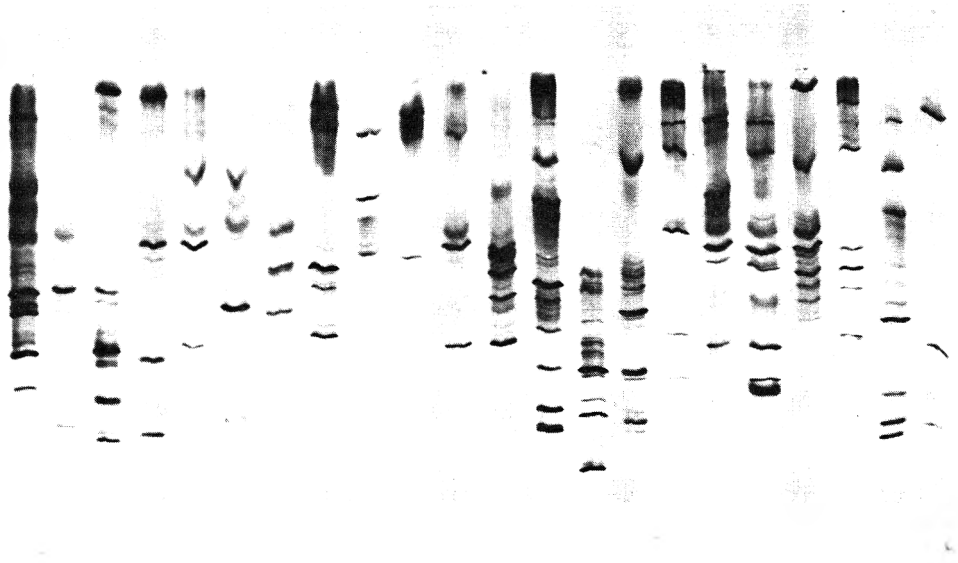

Figure 1. Sarcoplasmic protein patterns from 22 species of fish obtained from isoelectric focusing of samples in pH gradient 5-8 on agarose. Anode is at top of photograph.

From left to right: whiting (*Sillago maculata*), sea mullet (*Mugil cephalus*), dart (*Trachinotus russelli*), black bream (*Girella tricuspidata*), hussar (*Lutjanus amabilis*), John Dory (*Zeus faber*), jewfish (*Johnius antarctica*), barramundi (*Lates calcarifer*), thread finned salmon (*Eleutheronema tetradactylum*), king salmon (*Polydactylus sheridani*), coral trout (*Plectropoma maculatum*), bream (*Acanthopagrus australis*), tailor (*Pomatomus saltratrix*), tuna (*Kishinoella tonggol*), sweetlip (*Lethrinus chrysostomus*), parrot (*Choerodon venustus*), Moses perch (*Lutjanus russelli*), pearl perch (*Glaucosoma scapulare*), red emperor (*Lutjanus sebae*), spotted cod (*Cephalopholis cyanostigma*), schnapper (*Chrysophrys auratus*), and Spanish mackerel (*Cybium commersoni*).

(c) *Ampholine solution.*—pH 5-8. Pharmalyte pH 5-8 (Pharmacia Fine Chemicals AB).

(d) *Fixing solution.*—Dissolve 25 g sulfosalicylic acid (Ajax Chemicals) and 50 g trichloroacetic acid (Ajax Chemicals) in water and dilute to 500 mL with water.

(e) *Destaining solution.*—Mix 700 mL alcohol and 200 mL acetic acid. Dilute to 2 L with water.

(f) *Staining solution.*—Dissolve 0.5 g Coomassie Brilliant Blue R250 (Pierce Chemicals) in 250 mL destaining solution.

(g) *Cathode solution.*—1M NaOH.

(h) *Anode solution.*—0.05M H_2SO_4.

Apparatus

(a) *Isoelectric focusing.*—LKB 2117 Multiphor (LKB—Produkter AB, Sweden).

(b) *Power supply.*—Constant power, voltage, current (150 W, 2000 V, 150 mamp) (TPS Pty Ltd, Australia).

(c) *Circulating cooling water supply.*—Multitemp containing water at a constant temperature of 4°C (LKB—Produkter AB).

(d) *Plastic backing sheet.*—240 × 125 mm to support gel (Gel Bond, FMC Corp.)

(e) *Gel casting frame.*—Internal dimensions of 225 × 115 mm with thickness of 3 mm. Frame is made of plastic (Pharmacia Fine Chemicals AB).

(f) *Leveling table.*—To ensure that gel produced is perfectly uniform (LKB—Produkter AB).

(g) *Trays.*—300 × 200 × 30 mm to fix, stain, and destain gels.

(h) *Densitometer.*—Camag TLC/HPTLC scanner (Camag, Muttenz, Switzerland).

Sample Preparation

Obtain sarcoplasmic protein solution by macerating 10 g muscle tissue with 100 mL water at room temperature and then leaving overnight at 4°C. Centrifuge resulting solution at 9000 × g for 10 min at room temperature and use supernatant liquid obtained for analysis.

The mention of any product by trade name or the naming of commercial firms does not imply endorsement by The Government Chemical Laboratory.

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Gel Preparation

Pour water (2 mL) onto perfectly horizontal leveling table and place plastic backing sheet on table. Backing sheet is specially coated for "hydrophobic" side and a "hydrophilic" side.

Place "hydrophobic" side in contact with water on leveling table and remove excess water and air bubbles by rolling plastic sheet with prewetted glass rod. Carefully remove excess water with paper tissue.

Place plastic gel casting frame over leveling table and clamp with spring clips while ensuring that frame is inside confines of plastic backing sheet. Place table, sheet, and attached frame in 40°C oven 10 min to warm.

Mix agarose (0.3 g) and sorbitol (3.6 g) in conical flask with water (27 mL) and heat, with stirring, in boiling water bath until all solids are dissolved. Let solution cool to ca 75°C, and add 1.9 mL ampholine solution with shaking. Final solution is 30 mL, with ampholine concentration ca 2.5%, agarose concentration 1%.

Remove leveling table from oven, and pour agarose solution quickly and evenly over plastic backing sheet, being careful to avoid formation of air bubbles. Let gel set 15 min. Pass scalpel blade around interior of casting frame and carefully remove frame. Let gel harden 1 h at 4°C.

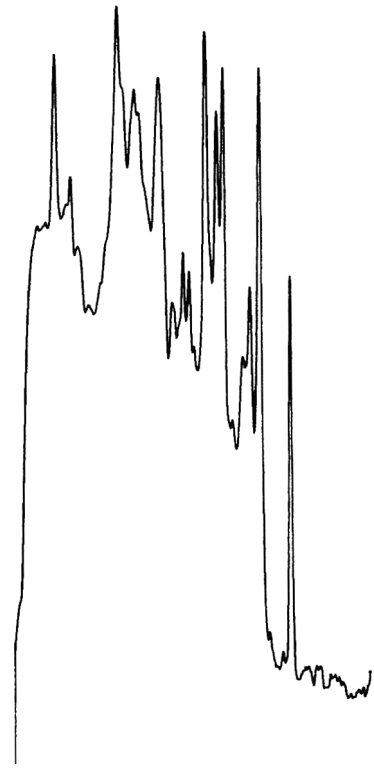

Figure 2. Typical densitometric scan (whiting).

Isoelectric Focusing

Smear water (2 mL) on cooling plate of electrofocusing apparatus and place plastic backing sheet and gel on plate, being careful to remove any air bubbles. Blot excess water from edges of plastic sheet by adsorbent paper toweling.

Soak filter paper strips (10 × 5 mm) in samples and apply to surface of gel near anode.

Thoroughly wet sponge rubber electrode strips with appropriate solutions for cathode and anode, blot on filter paper ca 1 min, and apply to edges of gel. Positioning of electrode strips corresponds with anode and cathode platinum wire contacts.

Supply isoelectric focusing apparatus with cooling water at 4°C from circulating water supply, and connect platinum electrode strips to constant power supply with 15 watts of constant power. Limit voltage to 1500 V maximum. Remove sample application strips after 45 min and continue separation to minimum of 1.5 h.

Fixing, Staining, and Destaining

After completing isoelectric phase of separation, remove gel and plastic backing sheet and place in tray containing fixing solution 30 min. Wash gel and backing sheet in destaining solution 30 min. Dry gel by placing filter paper, glass plate, and 1 kg weight over it 15 min followed by final drying with a draught of hot air from hand-held hair dryer.

Stain gel in staining solution 10 min. Place stained gel in destaining solvent until background is clear. Remove, drain, and finally dry with hair dryer.

Densitometry

Scan dried gel on plastic backing at wavelength of 577 nm by densitometer, using slit width of 0.1 mm, slit length of 1 mm, and scanning speed of 1 mm/s. Feed output from scanner to chart pen recorder with 10 mV full scale deflection.

Results and Discussion

The results obtained from the analysis of different species of fish are shown in Figure 1 with the anode (pH 5) at the bottom and the cathode (pH 8) at the top. The patterns are unique and are far less complicated than the patterns which

would have been obtained had a wide range (pH 3–10) ampholine been used.

The patterns obtained are highly reproducible, and unknown samples have been readily identified. It is, of course, conceivable that one species of fish will have no characteristic bands in this interval but experience to date with fish protein subjected to isoelectric focusing shows that this is not the case. All species of fish examined in this laboratory have characteristic bands in this interval.

The pH gradient was measured with a surface pH electrode and was found to be uniform, though this is not done on a routine basis. The use of a visible tracker protein is not deemed to be necessary.

The gel was subjected to densitometric scanning and the results for one sample, whiting, are shown in Figure 2. The slit width and scanning speed chosen produced adequate resolution and led to unique and highly reproducible patterns. Unknown samples were readily identified by comparison with the densitometer scans of standards. Traces of protein from the same fish may differ slightly in intensity or sharpness of the bands but never in overall pattern.

Experience has shown that agarose has definite advantages in time, ease, and safety when compared with polyacrylamide. The quality and reproducibility of the patterns obtained with agarose are equivalent to polyacrylamide in the pH interval studied (pH 5–8).

Densitometric scanning of the protein patterns obtained in the visible region in conjunction with visual comparisons offers an unequivocal means of fish species identification.

Because of the added ease and the time saved, it is suggested that agarose be used as a support medium in isoelectric focusing in preference to polyacrylamide for fish species identification.

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References

(1) Weime, R. J. (1965) *Agar Gel Electrophoresis*, Elsevier, Amsterdam, The Netherlands
(2) Nee, T. W. (1975) *J. Chromatogr.* **105,** 231–249
(3) Bai, S. G., & Radola, B. J. (1977) *Chem. Mikrobiol. Technol. Lebensm.* **5,** 33–37
(4) Tinbergen, B. J., & Olsman, W. J. (1976) *Fleischwirtschaft* **10,** 1501–1504
(5) Lundstrom, R. C. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 624–629
(6) Lundstrom, R. C., & Roderick, S. A. (1979) *Sci. Tools* **26**(3), 38–43

FERTILIZERS

Sulfur Oxidation in Fertilizers using Nitric-Perchloric Acid

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AOAC 2.160-2.161 is inadequate for determining total sulfur in fertilizers. The bromine-nitric acid combination does not oxidize all reduced forms of sulfur to sulfate; consequently, some findings are biased low. A perchloric-nitric acid mixture was used as the oxidizing mixture, and recovery of sulfur from 3 standards was complete. Also, quantitative results on fertilizer samples compared favorably with a potassium permanganate-nitric acid procedure that has been successfully used in the authors' laboratory for about 16 years. The described method is recommended for determining all forms of sulfur found in fertilizers except elemental sulfur. A procedure is described for determining elemental sulfur.

The determination of sulfur by precipitation as barium sulfate ($BaSO_4$) has long been recognized as an accurate means of sulfur determination. Textbooks (1, 2) have described the method of preparing and collecting the $BaSO_4$ precipitate. In 1959, Morris and Bozalek (3) reported a method developed by means of a statistical approach for the determination of sulfate in superphosphate fertilizers. Their method involved a simple hydrochloric acid digestion step; however, they recognized that certain ions interfered with their procedures. Barker (4) adapted the Morris and Bozalek method for the determination of total sulfur in fertilizers. He added steps to oxidize the sulfur and to remove nitrate, fluoride, and silica. His digestion procedures involved the addition of bromine in carbon tetrachloride, nitric acid, and HCl to the fertilizer samples. The method was adopted by the AOAC as official first action in 1964 (5). However, this method, AOAC **2.160-2.161** (6), is inadequate for determining sulfur in fertilizers (7) because the recommended bromine-nitric acid oxidizing mix does not completely convert some reduced sulfur compounds to sulfate.

Because of the inadequacy of **2.160-2.161** (6), in 1965 we began using a nitric acid-potassium permanganate (HNO_3-$KMnO_4$) oxidizing mix for determining total sulfur. In this procedure, the chemist must observe any insoluble yellow elemental (free) sulfur floating on the digest. This free sulfur is analyzed and added to sulfate sulfur to obtain accurate total sulfur results.

Initially, we attempted to oxidize various forms of sulfur with sodium peroxide. This one-step oxidation worked well for almost all fertilizers, especially the thiosulfates that had consistently required 2 steps when oxidizing with HNO_3-$KMnO_4$. However, 2 samples received from the regulatory service were repeatedly found to be lower in sulfur with sodium peroxide than with HNO_3-$KMnO_4$. However, Thorpe (7) successfully used peroxides for analyzing sulfur; her method was adopted official first action in 1980 (8).

Sulfur analysts agree on the need for a single method of oxidizing all forms of sulfur to sulfate. Therefore, a strong oxidizing mixture of nitric acid-perchloric acid (HNO_3-$HClO_4$) was investigated. The described method resulted from this investigation.

METHOD

Reagents and Apparatus

(a) *Potassium permanganate solution (saturated).*—Weigh 5 g $KMnO_4$ into 100 mL volumetric flask. Dilute to volume with water and mix.

(b) *Barium chloride solution.*—Dissolve 100 g $BaCl_2.2H_2O$ in 900 mL water and filter through Whatman No. 42 paper (**2.160(a)**).

(c) *Ammonium hydroxide solution.*—Add 50 mL NH_4OH to 50 mL water and mix.

(d) *Methyl orange indicator solution.*—Dissolve 0.1 g in 100 mL water.

(e) *Gooch crucible.*—50 mL, high form, with medium porosity fritted disc.

Sample Preparation and Determination

HNO_3-$HClO_4$ oxidizing method (see **2.020**(e) (9)).—Weigh 1 g fertilizer into 200 mL volumetric flask. Add ca 25 mL water and swirl until mixed. Add 25 mL HNO_3, boil gently ca 30 min (until solution turns straw yellow or clear), and cool. Add 15 mL 70-72% $HClO_4$, boil very

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Table 1. Recoveries of sulfur from chemicals with HNO_3–$HClO_4$

Chemical	Guarantee	Recoveries, %[a]		
		Mean	SD	CV, %
Na_2SO_4	22.57	22.63	0.15	0.66
Na_2SO_3	25.44	25.36	0.15	0.59
$Na_2S_2O_5$	33.73	33.55	0.38	1.13
$Na_2S_2O_3$	40.55	40.41	0.31	0.77

[a] Based on 5 replicates.

gently until solution is colorless or nearly so and dense white fumes appear in flask. Danger! Do *not* boil to dryness at any time. If samples contain large amounts of organic matter, increase temperature to fuming point, ca 170°C, over a period of ≥1 h. Cool slightly, add 50 mL water, and bring to a gentle boil. Cool and fill to volume with water. Let settle ≥4 h (or filter through paper). Transfer aliquot containing 50–150 mg S into 250 mL graduated beaker and dilute to ca 150 mL with water. Add methyl orange indicator until pink holds while stirring (ca 3 drops). Add, dropwise, NH_4OH solution until yellow holds when stirred. Add ca 1 mL HCl (ca pH 1). According to **2.161** (6), heat filtrate to boiling. Add 5–6 drops $BaCl_2$ solution. After 1 min, add, dropwise, an amount of $BaCl_2$ solution equivalent to expected S content plus 5 mL excess. Digest at gentle boil 1 h. Remove from hot plate and let precipitate settle 15–20 min. Filter immediately through previously ignited and weighed gooch. Wash with hot water until 10 mL wash water shows no precipitate with 3 mL 1% $AgNO_3$. Dry and ignite at 800°C to constant weight. Cool in desiccator over $MgClO_4$ and weigh.

$$\% \text{ S} = \text{g } BaSO_4 \times 2747.4/\text{g sample} \times \text{mL aliquot}$$

HNO_3–$KMnO_4$ oxidizing method.—Weigh 1 g fertilizer into 200 mL volumetric flask. Add ca 10 mL water and swirl until mixed. Add, dropwise, $KMnO_4$ solution until violet remains after swirling. Add 10 mL HNO_3, boil gently ca 15 min (until solution turns straw yellow or clear), and cool slightly. Add 50 mL H_2O, bring to gentle boil, and cool slightly. If yellow precipitate (free S) is observed in flask, filter under vacuum through gooch, catching filtrate in 200 mL volumetric flask. Wash residue with water several times while applying vacuum (volume should not exceed 200 mL). Remove flask containing filtrate, cool, fill to volume with water, and let settle if needed. Proceed as described in preceding paragraph, "Transfer aliquot containing 50–150 mg S into . . .". Treat residue as described in following paragraph. If no yellow precipitate is observed, proceed as described in preceding paragraph, "Cool and fill to volume with water. Let settle . . .".

Free sulfur procedure.—Thoroughly wash residue from HNO_3–$KMnO_4$ oxidizing method 5 times with 5 mL filtered, S-saturated acetone and vacuum filter after each wash. Dry gooch and contents 1 h at 100°C, cool in desiccator, and weigh. Wash contents of gooch 5 times with CS_2 until all free S is removed. Vacuum-filter after each wash. Dry crucible and contents 1 h at 100°C, cool in desiccator, and weigh.

$$\% \text{ Free S} = \text{difference in wt of crucible and contents before and after washing with } CS_2 \times 100/\text{g sample}$$

Add free S results to HNO_3–$KMnO_4$ oxidizable S (from filtrate) to obtain total S. Samples containing only elemental sulfur should be analyzed by the free sulfur procedure.

Results and Discussion

For about 16 years, we successfully used the HNO_3–$KMnO_4$ method, in conjunction with the free sulfur procedure, for determining sulfur in fertilizers. However, during all those years we desired a consistent, one-step method. Therefore, an attempt was made to derive a one-step oxidation procedure.

We theorized that $HClO_4$, an excellent oxidizing agent, would completely oxidize all forms of sulfur found in fertilizers. Since a HNO_3–$HClO_4$ mix is used in AOAC **2.020**(e) to oxidize phosphorus compounds to phosphate, this mix was investigated.

Digestions were made with HNO_3–$HClO_4$ on sodium sulfate (Na_2SO_4), sodium sulfite (Na_2SO_3), sodium metabisulfite ($Na_2S_2O_5$), and sodium thiosulfate ($Na_2S_2O_3$). Recoveries were complete and were not significantly different from the guarantee (Table 1). Also, coefficients

Table 2. Comparison of oxidation procedures for determining sulfur in fertilizers received through the state control services

	HNO_3–$HClO_4$[a]		HNO_3–$KMnO_4$[a]	
Guarantee	Mean	SD	Mean	SD
1.0	3.11	0.09	3.21	0.22
4.0	5.01	0.07	4.77	0.17
5.0	5.01	0.06	4.54	0.22
9.0	10.26	0.33	10.39	0.11
14.0	16.17	0.12	16.38	0.56
26.0	26.03	0.31	25.70	0.88

[a] Based on 5 replicates.

of variation of approximately 1% were quite encouraging.

To compare the HNO_3–$HClO_4$ oxidation against the HNO_3–$KMnO_4$ oxidation, 3 dry and 3 liquid fertilizer samples from the state control service were analyzed. Equal aliquots were taken for precipitate formation. No significant differences for sulfur were found by the 2 methods (Table 2). Standard deviations tended to be greater for the HNO_3–$KMnO_4$ oxidation, especially for the liquid thiosulfates containing 26% sulfur, which required additional analysis of free sulfur.

Of the 4300 fertilizer samples that have been analyzed for sulfur since this initial investigation, over 400 have been analyzed by both the HNO_3–$HClO_4$ and HNO_3–$KMnO_4$ methods. No practical differences have been found to negate the validity of the initial findings.

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References

(1) Vogel, A. I. (1951) *Quantitative Inorganic Analysis*, 2nd Ed., Longmans, New York, NY, p. 401
(2) Kolthoff, I. M., & Sandell, E. B. (1952) *Quantitative Inorganic Analysis*, 3rd Ed., McMillan, New York, NY, pp. 322–330
(3) Morris, A. G. C., & Bozalek, S. J. (1959) *Anal. Chim. Acta* **21**, 215–221
(4) Barker, J. E. (1964) *J. Assoc. Off. Agric. Chem.* **47**, 436–438
(5) Smith, J. B., Lay, H. W., & Marshall, C. V. (1964) *J. Assoc. Off. Agric. Chem.* **47**, 120
(6) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **2.160–2.161**
(7) Thorpe, V. A. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 854–858
(8) "Changes in Methods" (1980) *J. Assoc. Off. Anal. Chem.* **63**, 374–375
(9) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, sec. **2.020(e)**

FRUITS AND FRUIT PRODUCTS

Comparison of Enzymic, Gas-Liquid Chromatographic, and High Performance Liquid Chromatographic Methods for Determining Sugars and Organic Acids in Strawberries at Three Stages of Maturity

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Free sugars and major nonvolatile organic acids present in strawberries at 3 degrees of ripeness were determined by 3 analytical methods: enzymic, gas-liquid chromatographic, and high performance liquid chromatographic. Results showed that variability in sugar composition due to both degree of ripeness and method of analysis was greater for sucrose than for glucose and fructose. Sucrose was almost completely hydrolyzed in the overripe fruit. Acid results showed that there was little variation in citric acid levels due to ripeness or method of analysis; malic acid, however, decreased greatly in overripe fruit. Malic acid also showed high variability due to method of analysis. The glucose:fructose ratios for the underripe, ripe, and overripe fruit were 0.86, 0.92, and 0.60, respectively. The citric:malic ratios were 1.58, 2.39, and 14.86 for the underripe, ripe, and overripe stages, respectively.

Sugars and acids are major components of fruits, which have a lower susceptibility to change during processing and storage than other components such as pigments and flavor compounds. This relative stability offers a practical advantage for using sugar and nonvolatile acid profiles as an index of authenticity in products such as juices and juice concentrates.

Fructose, glucose, and sucrose are the main sugars reported in strawberries (1-6). Wrolstad and Shallenberger (7) found considerable variation in the free sugar composition reported in the literature for strawberries. Dako et al. (2), Lee et al. (5), and Selvaraj et al. (8) found differences in quantitative composition among varieties; Sweeney et al. (4) examined the seasonal as well as varietal variation of free sugars. Presence of the sugar alcohols sorbitol (9) and xylitol (9, 10) has also been reported. The major acids of strawberries are citric and malic; lesser amounts of glucolic, succinic, fumaric, isocitric, galacturonic, aspartic, glutamic, phosphoric, and quinic acids have been reported (4, 11-13).

The purpose of this work was to determine the sugar and nonvolatile acid composition of strawberry fruit at 3 stages of maturity. An additional objective was to compare 3 analytical methods—gas-liquid chromatographic (GLC), high performance liquid chromatographic (HPLC), and enzymic methods—in the quantitative determination of sugars and nonvolatile acids.

Experimental

Plant Material

Strawberries (*Fragaria ananassa* Duch. variety Benton) at 3 visual degrees of ripeness—underripe, ripe, and overripe—were obtained from the OSU Department of Horticulture during the 1979 harvest season. Fruits at each degree of ripeness were packed in polyethylene bags, quick-frozen at −34°C, and stored at −10°C.

Apparatus

(a) *Gas chromatograph.*—Varian Aerograph Model 200 with hydrogen flame ionization detector and 3 m × 2 mm id glass column containing 5% SE-52 on 80-100 mesh Chromosorb W(HP); injection temperature 190°C, detector temperature 275°C, nitrogen carrier gas flow 25 mL/min. For sugars, operate column isothermally 5 min at 165°C, program at 7°/min to 275°C, and hold at this temperature. For acids, program from 100 to 275°C at 7°/min and hold.

(b) *Liquid chromatograph.*—Varian Model 5000 equipped with column heater. For sugars: Varian Aerograph refractive index (RI) detector, 7.8 × 300 mm Aminex HPX-87 carbohydrate column (Bio-Rad Laboratories, Richmond, CA), column temperature 85°C, mobile phase 0.01% $CaCl_2 \cdot 2H_2O$ in degassed, deionized water filtered through 0.45 μm Millipore filter (keep at 65°C

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and continuously degas by use of magnetic stirrer), flow rate 1 mL/min, injection volume 20 μL. For acids: Varian liquid chromatographic variable wavelength detector Model UV-50 at 210 nm, 7.8 × 300 mm Aminex HPX-87 organic acid column (Bio-Rad Laboratories), column temperature 65°C, mobile phase 0.013N H_2SO_4 in deionized water filtered through 0.45 μm Millipore filter (keep at 65°C and continuously degas by use of magnetic stirrer), flow rate 0.8 mL/min, injection volume 20 μL.

(c) *Integrator.*—Hewlett-Packard Model 3380 A recording integrator.

(d) *Ultrasonic probe.*—Bronwill Biosonik III ultrasonicator; dial setting 70.

(e) *Spectrophotometer.*—Perkin-Elmer Model 550.

(f) *Syringes.*—For HPLC analyses: Glenco micro syringe, 50 μL capacity (Glenco Scientific, Inc., Houston, TX). For GLC analyses: Hamilton microliter syringe, 10 μL capacity (Hamilton Co., Reno, NV).

Reagents

(a) *Enzymic analysis.*—Glucose/fructose, sucrose/glucose, L-malic acid, and citric acid kits (Boehringer Mannheim Biochemicals, Indianpolis, IN). For isocitric acid, reagents specified by Boehringer Mannheim were obtained from that company.

(b) *Sugars and acids standards.*—β-D-Fructose crystalline (Sigma Chemical Co.); D-glucose certified ACS (Fisher Scientific Co.); sucrose Grade I crystalline (Sigma Chemical Co.); L-malic acid crystalline (Sigma Chemical Co.); citric acid monohydrate crystal reagent (Baker Chemical Co.); tartaric acid NF fine granular (Mallinckrodt Chemical Works).

Sugars and acids were stored under vacuum over P_2O_5 at least 24 h before weighing.

(c) *Ion-exchange resins.*—Wash 12 mL cationic-exchange resin (Bio-Rad AG 50W-X4, 200–400 mesh in hydrogen form) with 25 mL deionized water. Wash 12 mL anionic-exchange resin (Bio-Rad AG 1-X8, 200–400 mesh, acetate form) with 25 mL 0.1N acetic acid and then with 25 mL deionized water.

(d) *Polyvinylpyrrolidone polymer (PVPP).*—Wash PVPP according to procedure described by Loomis (14).

Sample Preparation

Weigh 250 g whole frozen fruit, heat to boiling in microwave oven, hold 30 s in boiling water bath, and cool immediately by immersing in ice bath. Correct weight loss by adding water. Homogenize in Waring blender. For GLC analyses, weigh 25.00 g; for HPLC analyses, weigh 10.00 g; for enzymic analysis, weigh 5.00 g homogenate. Store samples at −10°C for further analysis.

Determination

(a) *Sugars and acids by GLC.*—Transfer 25.00 g prepared sample homogenate to centrifuge tube, add 75 mL 95% ethanol, ultrasonicate 3 min, and centrifuge at 2000 × g for 10 min. Wash residue twice with 25 mL 80% ethanol, and combine supernates. Percolate extract through cationic-exchange column and then through anionic-exchange column. Wash columns with deionized water until 1 L is eluted. For sugar analysis, take 100 mL of fraction eluted from columns and dilute with water to 250 mL. Pipet 1 mL into 3 mL vial containing 100 μL 0.2% rhamnose in 80% ethanol as internal standard. Dry sample on rotary evaporator (35°C) and store under vacuum over P_2O_5 at least 24 h.

Recover acids from anionic column by washing with 250 mL 10N formic acid, followed with deionized water until 1 L eluate is collected. Take 100 mL aliquot of acid fraction to dryness on rotary evaporator (40°C) and dissolve residue in 10 mL water. Pipet 1 mL solution into 3 mL vial containing 100 μL 1% tartaric acid in 80% ethanol as internal standard. Take sample to dryness on rotary evaporator and store under vacuum over P_2O_5 at least 24 h.

(b) *Preparation of TMS derivatives.*—To sugars in 3 mL vials, add 0.3 mL Tri-Sil reagent (Pierce Chemical Co., Rockford, IL), shake vigorously in Buchler shaker 5 min, heat 20 min at 70°C in Pierce Reacti-Therm heating module, shake additional 15 min, and centrifuge. Inject 2 μL supernate into gas chromatograph.

To acids in 3 mL vials, add 0.3 mL Tri-Sil reagent, shake vigorously in Buchler shaker 5 min, heat 30 min at 50°C in Pierce Reacti-Therm heating module, shake additional 5 min, and centrifuge. Inject 2 μL supernate into gas chromatograph.

(c) *Sugars and acids by HPLC.*—For sugars, transfer 10.00 g prepared sample homogenate to centrifuge tube, add 40 mL 95% ethanol, and ultrasonicate 3 min. Refrigerate sample (2°C) 1 h, and then filter by suction through pad of 10 g hydrated PVPP on 7.0 cm diameter Whatman No. 1 paper. Rinse PVPP and sample residue 3 times with 25 mL portions of 80% ethanol. Combine filtrate and washings and concentrate under vacuum in rotary evaporator (35°C) to final volume of ca 5 mL. Percolate concentrate through

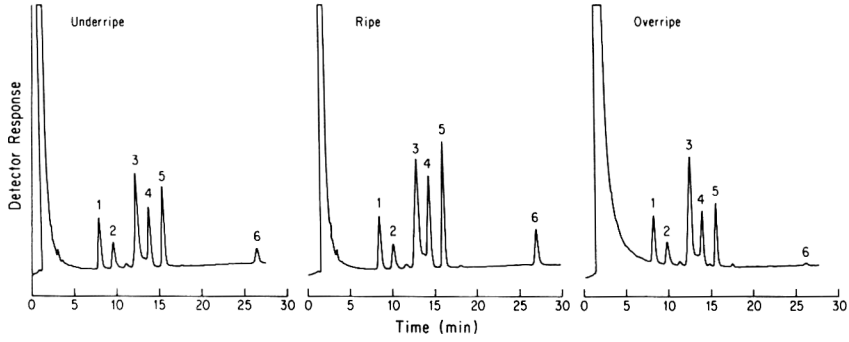

Figure 1. GLC separation on SE-52 column of TMS ethers of sugars from strawberry fruits at 3 degrees of ripeness: 1, and 2, rhamnose (int. std); 3, fructose; 4, α-glucose, 5, β-glucose; and 6, sucrose.

6 mL cationic-exchange column and then through 6 mL anionic-exchange column, and wash columns with deionized water. Collect all eluates from columns in 50 mL volumetric flask containing 5 mL 10% D-mannitol as internal standard and 1.0 mL 0.5% $CaCl_2 \cdot 2H_2O$. Filter sample through 0.45 μm Millipore filter before injection into HPLC apparatus.

For acids, proceed as previously described for sugars, but percolate solution only through 10 mL cation-exchanger (hydrogen form) and rinse with deionized water to final volume of 100 mL.

(d) *Sugars and acids by enzymic analyses.*—Determine sugar content with glucose/fructose and sucrose/glucose kits, following supplier's directions. Determine citric, isocitric, and malic acid content with enzyme kits, reagents, and recommended procedures of supplier.

Calculations

(a) *GLC analyses.*—Calculate individual sugar and acid content in sample according to following formulas:

$$\text{Sugar (g/100 g fruit)} = (PA/PA') \times (W'/K) \times (DF/R) \times 4$$

Two glucose peaks appear in the chromatogram; therefore, glucose peak area is sum of the α- and β-peaks.

$$\text{Acid (mequiv./100 g fruit)} = (PA/PA') \times (W'/K) \times (DF/R) \times (4/\text{equiv. wt})$$

where PA and PA' = peak area of sugar or acid in sample and standard, respectively; W' = weight of internal standard added to vial; K = detector response factor for given sugar or acid calculated as $K = (PA/PA')/(W/W')$; DF = dilution factor; and R = percent recovery.

(b) *HPLC analyses.*—Calculate content of individual sugar by the following formula:

$$\text{Sugar (g/100 g sample)} = (PA/PA') \times (W'/K) \times (DF/R) \times 10$$

Results and Discussion

Strawberries at 3 different visual degrees of ripeness were selected to study the influence of maturity on the variability in sugar and organic acid composition. The samples presented the following values for total soluble solids (°Brix) and titratable acidity (mequiv./100 g fruit): 6.0 and 199.8, 7.4 and 123.5, and 6.2 and 117.5 for underripe, ripe, and overripe fruit, respectively.

Figure 1 shows a typical GLC separation for strawberry sugars at each degree of ripeness. Major sugars present in all samples were fructose, glucose, and sucrose. The small peak present between α- and β-glucose in the GLC chromatogram of the overripe sample had the same retention time as sorbitol, suggesting that sor-

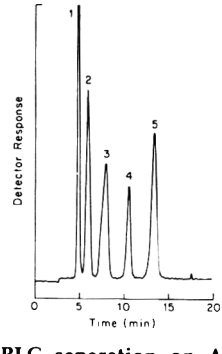

Figure 2. HPLC separation on Aminex HPX-87 carbohydrate column of free sugars from underripe strawberry fruit: 1, solvent; 2, sucrose, 3, glucose, 4, fructose; and 5, mannitol (int. std).

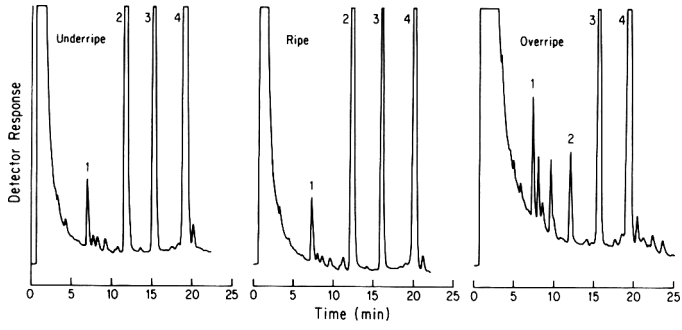

Figure 3. GLC separation on SE-52 column of TMS ethers of nonvolatile acids from strawberry fruits at 3 degrees of ripeness: 1, unknown; 2 malic; 3, tartaric (int. std); and 4, citric.

bitol could have been formed during the process of maturation. If this peak is sorbitol, its concentration would be approximately 200 μg/g fresh fruit or 0.78% of total sugars as estimated by its peak area. Makinen and Soderling (9) reported that ripe strawberries contain 320 μg sorbitol/g fresh weight, which would be 0.58% of total sugars if one assumes a total sugar content in strawberries of 5.5 g/100 g fresh weight (7). In an earlier publication (15), we stated that presence of sorbitol in strawberry products would be indicative of adulteration with less expensive sorbitol-containing fruits, such as fruits of the *Rosaceae* family—apples, pears, and plums. This suggestion needs to be qualified so that quantities of sorbitol in excess of trace amounts, e.g., 1% of total sugars, would be indicative of adulteration.

Figure 2 shows a typical HPLC chromatogram for separation of strawberry sugars. The time required for HPLC separation was 15 min, whereas that for GLC separation was 30 min. Derivatization of sugars for GLC analysis adds considerably to the total analysis time. Both the GLC and HPLC chromatograms reveal the cleanup achieved in sample preparation. Data on sugars by GLC are consistently low, but GLC offers greater sensitivity and better resolution.

Figures 3 and 4 show typical GLC and HPLC separations of the nonvolatile acids of strawberry fruit, which give results similar to other publications (4, 11, 12); citric and malic are the major acids present. It is evident that the minor compounds present in the underripe and ripe samples increased in concentration at the overripe stage. Further work needs to be done for identification and quantification of these minor compounds.

To determine the percent of recovery, known amounts of sugars and acids were subjected to the same extraction procedure as the samples. There was no evidence of sucrose hydrolysis when sucrose standards were subjected to preparation and separation conditions similar to those of the samples. Table 1 shows the detector response

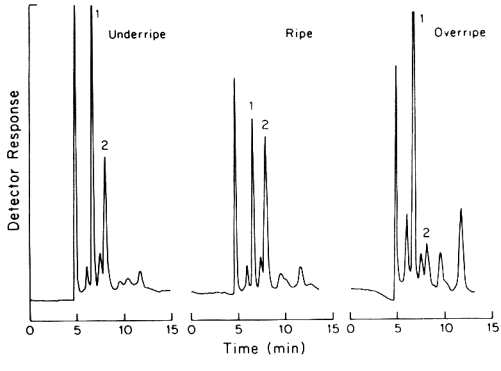

Figure 4. HPLC separation on Aminex HPX-87 organic acid analyses column of nonvolatile acids from strawberry fruits at 3 degrees of ripeness: 1, citric; and 2, malic.

Table 1. Detector response factor (*K*) and percent recovery of sugars and nonvolatile organic acids of strawberry

Sugar or acid	*K*	Rec., %	Method of anal.
Fructose	0.82	96.0	GLC
	1.04	95.5	HPLC
Glucose	1.56	100.0	GLC
	1.03	98.7	HPLC
Sucrose	1.14	100.0	GLC
	1.01	99.4	HPLC
Citric	0.80[a]	80.3	GLC
	ext std	95.2	HPLC
Malic	1.13[a]	82.3	GLC
	ext std	98.4	HPLC

[a] Reported by Akhavan et al. (16).

Table 2. Free sugars content of strawberry fruit (mean values)[a]

Degree of ripeness	Fructose g/100 g	Fructose % CV	Fructose % TS	Glucose g/100 g	Glucose % CV	Glucose % TS	Sucrose g/100 g	Sucrose % CV	Sucrose % TS	Total sugars	Glu/fru	Meth. of anal.
Underripe	1.48	3.23	50.17	1.24	2.94	42.03	0.23	7.84	7.80	2.95	0.84	ENZ
	1.23	5.47	50.62	1.08	6.47	44.44	0.12	11.03	4.94	2.43	0.88	GLC
	1.52	3.00	49.03	1.32	3.35	42.58	0.26	3.99	8.39	3.01	0.87	HPLC
Mean	1.41	3.9	49.94	1.21	4.25	43.02	0.20	7.62	7.04	2.80	0.86	—
Ripe	1.97	0.97	46.24	1.75	2.51	41.08	0.54	2.90	12.68	4.26	0.89	ENZ
	1.63	8.46	45.66	1.64	5.01	45.94	0.30	5.01	8.40	3.57	1.00	GLC
	2.22	3.84	47.03	1.94	1.77	41.10	0.56	3.99	11.86	4.72	0.87	HPLC
Mean	1.94	4.42	46.31	1.78	3.10	42.71	0.47	3.97	10.98	4.18	0.92	—
Overripe	1.83	3.99	62.03	1.07	1.96	36.27	0.05	24.36	1.69	2.95	0.58	ENZ
	1.55	2.32	61.26	0.97	3.98	38.34	0.02	18.39	0.40	2.53	0.63	GLC
	1.88	2.44	62.46	1.10	1.10	36.54	0.03	20.94	1.00	3.01	0.59	HPLC
Mean	1.75	2.97	61.92	1.05	2.35	37.05	0.03	21.23	1.03	2.83	0.59	—

[a] % CV = % coefficient of variance between duplicate samples.
% TS = percent total sugars (fructose + glucose + sucrose).
Glu/fru = Glucose:fructose ratio.
ENZ = enzymic, GLC = gas-liquid chromatographic, HPLC = high performance liquid chromatographic.

factors (K) and the percent recovery values used for quantitative purposes for the GLC and HPLC analyses. In the case of the HPLC analyses for citric and malic acids, a standard curve relating area and concentration was used for the quantitative analyses.

Table 2 shows the sugar content of strawberry fruits at 3 degrees of ripeness and by 3 methods of analyses. The percent coefficient of variance (% CV) indicates the variability between duplicate samples. In general, the % CV was lower for enzymic and HPLC analyses than for GLC analyses. Also, the quantitative values for the 3 sugars as determined by HPLC and enzymic methods were in closer agreement than GLC.

In general, maximum fructose, glucose, and sucrose content was obtained for ripe strawberry fruit. Fructose, glucose, and sucrose content agrees with values previously reported in the literature (1, 2, 5, 7). Sucrose content decreased dramatically at the overripe stage, probably because of enzymic hydrolysis. (The purpose of the microwave heating in sample preparation was to inactivate any native invertase.) Glucose:fructose ratio for the underripe and ripe samples showed a mean value of 0.89 which is close to an invert sugar pattern. In the overripe sample, the decrease in glucose was greater than the decrease in fructose, resulting in a glucose: fructose ratio of 0.60. Similar results were reported by Kliewer (17) in grapes.

Maltose was not found in any of our samples.

Table 3. Nonvolatile organic acids in strawberry fruit (mean values)[a]

Degree of Ripeness	Citric meq. 100 g	Citric % CV	Citric % TA	Isocitric meq. 100 g	Isocitric % CV	Isocitric % TA	Malic meq. 100 g	Malic % CV	Malic % TA	Total acids, meq./100 g	Citric: malic	Meth. of anal.
Underripe	13.5	0.33	64.71	0.46	7.70	2.20	6.93	0.48	33.09	20.9	1.96	ENZ
	10.4	2.38	60.26	—	—	—	6.89	1.59	39.73	17.3	1.52	GLC
	10.9	6.12	55.80	—	—	—	8.65	6.25	44.20	19.5	1.26	HPLC
Mean	11.6	2.94	60.26	—	—	—	7.49	2.77	39.01	19.1	1.58	—
Ripe	10.3	2.31	81.86	0.29	28.1	2.31	1.99	1.70	15.83	12.6	5.17	ENZ
	7.7	2.74	57.12	—	—	—	5.78	1.44	42.88	13.5	1.33	GLC
	5.8	3.64	40.26	—	—	—	8.65	6.32	59.74	14.5	0.67	HPLC
Mean	7.9	2.90	59.75	—	—	—	5.47	3.15	39.48	13.4	2.39	—
Overripe	10.2	0.74	91.94	0.23	7.80	2.08	0.66	1.82	5.98	11.1	15.38	ENZ
	12.1	2.81	96.25	—	—	—	0.47	6.43	3.75	12.6	25.63	GLC
	9.3	1.39	77.95	—	—	—	2.63	2.91	22.05	11.9	3.54	HPLC
Mean	10.5	1.65	88.71	—	—	—	1.25	3.72	10.59	11.8	14.85	—

[a] % CV = % coefficient of variance for duplicate samples.
meq. = milliequivalents.
% TA = % total acids.
ENZ = enzymic, GLC = gas-liquid chromatographic, HPLC = high performance liquid chromatographic.

Lee et al. (5) and Richmond et al. (6) reported the presence of maltose in their strawberry samples. Both authors refluxed their samples with 80% ethanol for at least 2 h during the sugar extraction. This practice could have promoted the hydrolysis of starch.

Table 3 shows the citric and malic content of strawberry fruits at 3 degrees of ripeness and by 3 methods of analysis. Also reported is the isocitric acid content determined by enzymic analysis at the 3 degrees of ripeness. The variability of the results within each method of analysis is quite acceptable; 6% CV is the highest variation. HPLC analyses showed the highest % CV and enzymic analyses the lowest. The agreement is not as good among the different methods as would be desired, however. HPLC determinations gave higher values for malic acid, particularly for the overripe sample. A possible source of error could be the presence of interfering compounds which absorb at 210 nm. GLC and enzymic determinations for malic acid were in fair agreement, except for the ripe sample. Values for citric, malic, and total acidity are in general accord with those reported by Sweeney et al. (4), Fernandez-Flores et al. (11), Ryan and Dupont (13), and Sistrunk and Cash (12). Because of the variability among methods, patterns for changes in acids during ripening are more clearly seen by examining either results for individual methods or means of the 3 methods. Table 3 reveals a decrease in total acidity during maturation. Citric acid levels were least at the ripe stage, with little difference in citric acid content between underripe and overripe fruit. In contrast, malic acid showed a large decrease at the overripe stage. These changes result in a much larger citric:malic ratio for overripe samples compared with underripe and ripe samples. In a previous study on the changes of nonvolatile acids during ripening of pears (18), similar results were reported: Malic acid content decreased sharply in overripe fruit whereas citric acid decreased only slightly. We were unable to quantitate isocitric acid by GLC and HPLC analyses because we could not satisfactorily resolve citric and isocitric acids. Enzymic analyses revealed that isocitric acid was present in strawberry fruit at very low concentration compared with citric or malic acid. Similar results for levels of isocitric acid in strawberry juice were reported by Benk (19).

We found less variation in the determination of sugars than in the determination of acids due to both analytical method and level of maturity. Variability of nonvolatile acid composition limits its use as an index of authenticity.

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References

(1) Hardinge, M. G., Swarner, J. B., & Crooks, H. (1965) *J. Am. Diet. Assoc.* **46,** 197–204
(2) Dako, D. Y., Trautner, K., & Somogyi, J. C. (1970) *Schweiz. Med. Wochenschr.* **100,** 897–903
(3) Kline, D. A., Fernandez-Flores, E., & Johnson, A. R. (1970) *J. Assoc. Off. Anal. Chem.* **53,** 1198–1202
(4) Sweeney, J. P., Chapman, V. J., & Hepner, P. A. (1970) *J. Am. Diet. Assoc.* **57,** 432–435
(5) Lee, C. Y., Shallenberger, R. S., & Vittum, M. T. (1970) "Free sugars in fruits and vegetables," New York's Food and Life Sciences Bulletin No. 1, State Agricultural Experiment Station, Geneva, NY
(6) Richmond, M. L., Brandao, S. C. C., Gray, J. I., Markakis, P., & Stine, C. M. (1981) *J. Agric. Food Chem.* 29(1), 4–7
(7) Wrolstad, R. E., & Shallenberger, R. S. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 91–103
(8) Selvaraj, U., Divakar, N. G., Suresh, E. R., Iyer, C. P. A., & Subramanyam, M. D. (1976) *J. Food Sci. Technol. India* **13,** 195–198
(9) Makinen, K. K., & Soderling, E. (1980) *J. Food Sci.* **45,** 367–371
(10) Washuttl, J., Riederer, P., & Bancher, E. (1973) *J. Food Sci.* **38,** 1262–1263
(11) Fernandez-Flores, E., Kline, D. A., & Johnson, A. R. (1970) *J. Assoc. Off. Anal. Chem.* **53,** 17–20
(12) Sistrunk, W. A., & Cash, J. N. (1973) *J. Food Sci.* 38, 807–809
(13) Ryan, J. J., & Dupont, J. A. (1973) *J. Agric. Food Chem.* **21,** 45–49
(14) Loomis, W. D. (1974) *Methods Enzymol.* **31,** 528–544
(15) Wrolstad, R. E., Cornwell, C. J., Culbertson, J. D., & Reyes, F. G. R. (1981) *Quality of Fruits and Vegetables of North America,* ACS Symp. Ser. No. 170, American Chemical Society, Washington, DC, p. 77-93
(16) Akhavan, I., Wrolstad, R. E., & Richardson, D. G. (1980) *J. Chromatogr.* **190,** 452–456
(17) Kliewer, W. M. (1965) *Am. J. Enol. Viticult.* **16,** 101–110
(18) Akhavan, I., & Wrolstad, R. E. (1980) *J. Food Sci.* **45,** 499–501
(19) Benk, E. (1978) *Alkohol Ind.* **91,** 88

DRUGS

High Pressure Liquid Chromatographic Determination of Physostigmine Salicylate and Physostigmine Sulfate in Liquids and Ointments: Collaborative Study

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Results of 11 laboratories are presented for the collaborative study of a proposed method for the quantitative reverse phase high pressure liquid chromatographic (HPLC) determination of physostigmine salicylate and physostigmine sulfate in pharmaceutical formulations. The samples consisted of commercial solution, injection, and ointment preparations, each containing one of the physostigmine salts. The physostigmine salt is extracted from ointments with acetonitrile after the ointment is dissolved in hexane. Liquid preparations are diluted directly. Physostigmine is determined at 254 nm on a C_{18} column by comparison with a physostigmine standard. Flurazepam hydrochloride is the internal standard. The method has been adopted official first action for the solution dosage form.

Physostigmine, also known as eserine, is the main alkaloid present in the seeds of the Calabar bean (*Physostigma venenosum*) (1). It is usually obtained by extracting the dried, ripe seeds with alcohol. Physostigmine's miotic effect makes it useful in ophthalmology in the treatment of glaucoma; it increases the facility of outflow of aqueous humor and thus reduces intraocular tension (2).

Physostigmine has been determined by acidimetric (3, 4), colorimetric (5), and ultraviolet (UV) spectrophotometric (6) techniques. The acidimetric procedures include both a back titration of excess acid with base in an aqueous system and a nonaqueous titration with perchloric acid in dioxane or acetic acid. The colorimetric procedure is based on the reaction of physostigmine with sodium nitrite in acid solution to form a yellow nitroso compound which is stabilized with ammonium sulfamate. In the UV spectrophotometric procedure, the physostigmine is extracted into an aqueous medium as the salt and its absorbance is measured at the wavelength of maximum absorbance at either 246 or 305 nm. All of these procedures are rather nonspecific and are not stability-indicating.

One of the characteristics of physostigmine is that it is easily hydrolyzed to eseroline, methylamine, and carbon dioxide. The eseroline, which is colorless, is further oxidized to rubreserine, which is red (7). Physostigmine products may turn red as they age, indicating a chemical deterioration. Thus, work was initiated to develop a more specific assay that would be stability-indicating.

High pressure liquid chromatography (HPLC) was investigated as a means to both identify and quantitate physostigmine and its salts. A reverse phase HPLC system using octadecyl-bonded silica microparticles as the stationary phase and acetonitrile–pH 6.0 ammonium acetate buffer as the mobile phase satisfies these requirements. This system separates physostigmine from its degradation products, as well as from preservatives that are frequently present.

Figure 1 shows one HPLC chromatogram of physostigmine salicylate, before and after degradation with sodium hydroxide, obtained by the proposed systems. Figures 2–4 show typical HPLC chromatograms of the samples used in this collaborative study. The degradation products and preservatives encountered all eluted before the physostigmine peak, which has a retention time of 3–4 min.

Physostigmine has a high molar absorptivity, 12140 in acetonitrile, with an absorbance maximum at about 256 nm. The UV detector at 254 nm was sufficiently close to the maximum absorbance to provide a good response.

A recovery of 99.2% was obtained for physo-

This report of the Associate Referee was presented at the 94th Annual Meeting of the AOAC, Oct. 20–23, 1980, at Washington, DC.

The recommendations of the Associate Referee were approved by the General Referee. Committee B approved the recommendations, except the method was approved only for the solution dosage form. The Committee's recommendation was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 65 (March issue).

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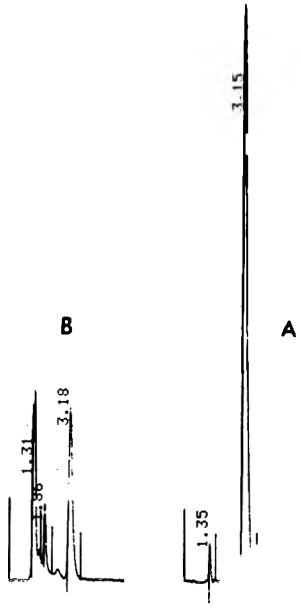

Figure 1. HPLC chromatogram of physostigmine salicylate, 0.1 mg/mL water, before degradation (A) and after partial degradation (B) with 2 drops of 1N NaOH.

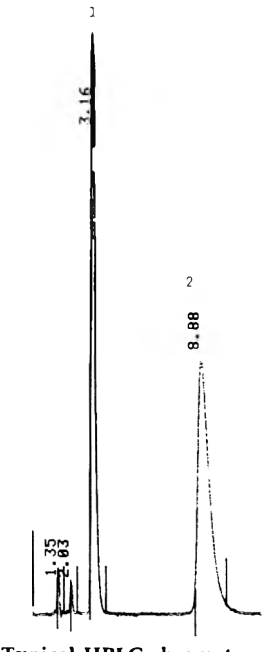

Figure 3. Typical HPLC chromatogram of physostigmine salicylate solution sample: 1, physostigmine salicylate; 2, flurazepam HCl internal standard.

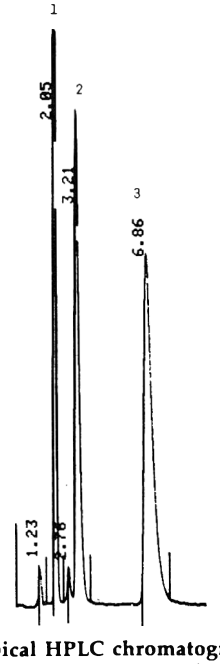

Figure 2. Typical HPLC chromatogram of physostigmine salicylate injection sample: 1, benzyl alcohol; 2, physostigmine salicylate; 3, flurazepam HCl internal standard.

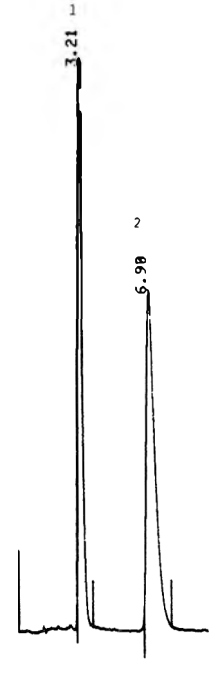

Figure 4. Typical HPLC chromatogram of physostigmine sulfate ointment sample: 1, physostigmine sulfate; 2, flurazepam HCl internal standard.

stigmine sulfate by the method for ointment preparation, which involved addition of 7.5 mg standard/g ointment.

Physostigmine salicylate and physostigmine sulfate were extracted from 20 mL hexane with two 20 mL portions of acetonitrile. The proposed HPLC method was used to compare the extracted standards with similar standard solutions that were not extracted. The amount of standard recovered through the extraction was 98.6% physostigmine salicylate and 100.4% physostigmine sulfate.

Physostigmine Salicylate and Physostigmine Sulfate-High Pressure Liquid Chromatographic Method

Principle

Physostigmine salicylate or sulfate is dissolved or extd into acetonitrile and detd as physostigmine by HPLC with UV (254 nm) detector and with flurazepam as internal std.

Apparatus

(a) *Liquid chromatograph.*—Model 204 equipped with 2 Model 6000 pumps, Model 660 solv. programmer, 254 nm UV detector, Model U6K injector (Waters Associates, Inc.) and Model 3380A integrator (Hewlett-Packard). Equiv. HPLC system with strip chart recorder may be used.

(b) *HPLC column.*—μBondapak C_{18}, 3.9 mm id × 30 cm (Waters Associates, Inc.) or equiv. reverse phase column providing appropriate retention times and sepn for physostigmine and internal std.

Reagents

(a) *Ammonium acetate.*—0.05M. Dissolve 3.85 g NH_4OAc in H_2O and dil. to 1 L. Filter thru 4.7 cm Whatman G F/F glass microfiber paper, or equiv., in Millipore-type filter holder. Adjust filtrate to pH 6.0 ± 0.1 with HOAc or NH_4OH.

(b) *Solvents.*—UV grade hexane and acetonitrile (Burdick & Jackson Laboratories, Inc., or equiv.) filtered thru same filter as in (a).

(c) *Mobile phase.*—Acetonitrile-0.05M NH_4OAc (1 + 1) at flow rate of ca 2.0 mL/min. Mobile phase ratio and flow rate may be varied to give approximate retention time of 3–4 min for physostigmine peak (first) and separation of flurazepam internal std peak (second).

(d) *Internal std soln.*—Dissolve 50 mg flurazepam HCl in MeOH and dil. to 100 mL with MeOH.

(e) *Physostigmine std solns.*—3.0 mg/100 mL. Transfer 60 mg accurately weighed USP Physostigmine, Physostigmine Salicylate, or Physostigmine Sulfate to 100 mL vol. flask and dil. to vol. with acetonitrile. Transfer 5.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln and dil. to vol. with acetonitrile. Use physostigmine and salicylate stds without drying. Dry sulfate std 2 h at 105°C.

Sample Preparation

(a) *Ointments.*—Transfer accurately weighed sample (W) contg ca 3 mg physostigmine salicylate or physostigmine sulfate to 60 mL separator. Add 20 mL n-hexane and ext with four 20 mL portions of acetonitrile. Collect exts in 100 mL vol. flask contg 5.0 mL internal std soln and dil. to vol. with acetonitrile.

(b) *Liquids.*—Transfer aliquot of liq. (V) contg ca 3 mg physostigmine or its salts to 100 mL vol. flask contg 5.0 mL internal std soln and dil. to vol. with acetonitrile.

System Suitability (Chromatographic System) Check

(a) *Reproducibility.*—Let system equilibrate with flow rate of ca 2 mL/min. Then make four 10.0 μL injections of any std soln. Measure coeff. of variation of peak response for 4 injections by following formula:

$$\text{CV, \%} = 100\sqrt{\frac{\sum(x-\bar{x})^2}{n-1}} \div \bar{x}$$

where x = ratio of area of physostigmine peak divided by area of internal std peak, $\bar{x}$ = mean of these ratios, and n = number of injections.

Coeff. of variation should be ≤2%. If reproducibility is unsatisfactory, let system equilibrate longer and repeat test.

(b) *Resolution.*—Retention time for physostigmine peak should be 2.5–4.5 min. Resolution factor, R, for physostigmine peak and internal std peak should be ≥3.0, using following formula:

$$R = 2(t' - t)/(PW + PW')$$

where t and t' = mm retention of physostigmine and internal std peaks, respectively; and PW and PW' = mm peak widths measured at baseline of physostigmine and internal std, respectively.

Determination

Make duplicate 10 μL injections each of sample soln and appropriate std soln, alternating sample and std solns. Calc. results by using response ratios (RR) rel. to internal std, based on peak areas:

For ointments:

Physostigmine (or salt), mg/g = $100 \times (RR/RR') \times (C/W)$

For liqs:

Physostigmine (or salt), mg/mL = $100 \times (RR/RR') \times (C/V)$

where RR and RR' = response ratio of sample and std; C = concn of std (mg/100 mL); W = g ointment; V = mL liq. Identification is based on same retention times for samples and stds.

Collaborative Study

A collaborative study was conducted in which 11 collaborators examined 3 samples. Table 1 lists the composition of each sample. Each collaborator was supplied with the 3 samples and 200 mg each of standard physostigmine salicylate, standard physostigmine sulfate, and internal standard flurazepam hydrochloride. Each sample consisted of intact, commercially prepared units from single lots. Both the commercially obtained physostigmine salicylate and physostigmine sulfate standards were analyzed by the author according to the *U. S. Pharmacopeia* (USP) XX monographs (4) for these compounds and failed the "Readily Carbonizable Substances" test. This was believed not to affect the results of the collaborative study. The physostigmine sulfate standard also failed the "Loss on Drying" test, being 1.8% more than the allowable limit. The standard is dried before use and this loss on drying would not affect the results.

Collaborators were instructed as to the amount of each sample to use to obtain the correct final concentration. The standard physostigmine sulfate was to be dried at 105°C to constant weight before use and the standard physostigmine salicylate was to be used without drying. The collaborators were instructed to keep all solutions in the dark until ready for use and to calculate peak areas by a recording integrator, if available.

Table 1. Composition of samples for collaborative study

Composition	Label content, %
Physostigmine Salicylate Solution	
Physostigmine salicylate	0.5
Chlorobutanol	0.15
Hydroxypropyl methylcellulose	0.5
Sodium chloride	
Citric acid	
Sodium bisulfite	
Purified water	
Physostigmine Salicylate Injection	
Physostigmine salicylate	1 mg/mL
Sodium bisulfite	0.1
Benzyl alcohol	2.0
Water for injection	
Physostigmine Sulfate Ointment	
Physostigmine sulfate	0.25[a]
Anhydrous lanolin	4
White petrolatum	75
Mineral oil	20
Purified water	1

[a] Personal communication from manufacturer of product.

Results and Discussion

Individual results from each collaborator are shown in Table 2, along with the average, range, and the coefficient of variation for each of the 3 samples. The results from Collaborator 4 could not be used, because the system suitability test for coefficient of variation was not met. Because Collaborator 9 made only one injection for the physostigmine salicylate solution and physostigmine sulfate ointment samples, only one result was reported. The results from Collaborator 6 for the physostigmine salicylate sample solution met Dixon's requirements for an outlier (8) and were discarded. This collaborator reported results that were approximately 15% lower than the mean for both the physostigmine salicylate solution and injection samples. Because the same standard solution was used for both samples, it appears that this collaborator could have made an error in the standard concentration.

The physostigmine salicylate solution was assayed by the author according to the USP XX (9) procedure. A result of 0.555% was obtained, compared with a mean of 0.479% by the HPLC procedure. The physostigmine sulfate ointment was also assayed by the USP XX procedure with a result of 0.254% compared with a mean of 0.238% by the HPLC method. The higher values obtained by the USP methods indicate their lack of specificity. Insufficient physostigmine salicylate injection was available for assay by the USP procedure, because the nonaqueous titration required a minimum of 50 mg, or, in this case, 25 ampules. The samples were not assayed by the AOAC methods for physostigmine; these methods were only general for alkaloids, which are extracted and titrated with 0.02N sulfuric acid. More than 20 times the amount of sample is needed for the titrimetric procedure as for the proposed HPLC method to produce a titration of at least 10 mL 0.02N sulfuric acid.

When reagent grade hexane was used to ex-

Table 2. Results of collaborative study for physostigmine

Coll.	Column	CV, %	Resolution	Retention, min Phys./flur.	Amount found: Solution, %	Amount found: Injection, mg/mL	Amount found: Ointment, %
1	μBondapak C_{18}	1.5	3.4	3.5/6.9	0.482	0.886	0.255
					0.477	0.862	0.245
2	μBondapak C_{18}	1.0	6.5	3.3/6.7	0.486	0.962	0.240
					0.486	0.952	0.243
3	μBondapak C_{18}	0.55	2.8	3.3/5.6	0.470	1.000	0.205
					0.477	0.992	0.203
4	μBondapak C_{18}	3.66[a]	6.2	3.8/8.0	0.595	0.988	0.247
					0.542	1.049	0.222
					0.449	1.019	0.256
					0.382	1.073	0.224
5	μBondapak C_{18}	0.79	5.6	3.7/6.4	0.492	0.939	0.244
					0.482	0.983	0.243
6	μBondapak C_{18}	0.37	2.8	3.4/5.6	0.417[b]	0.796	0.236
					0.417[b]	0.803	0.241
7	Ultrasphere ODS	1.73	4.33	3.5/11.2	0.466	0.962	0.226
					0.462	0.971	0.221
8	LiChrosorb RP-18	0.63	4.17	3.9/6.9	0.478	0.957	0.230
					0.466	0.974	0.227
9	Spherisorb S-ODS	0.67	2.84	4.0/6.7	0.476	0.950	0.248
						0.952	
10[c]	μBondapak C_{18}	0.58	3.2	3.2/7.0	0.508	1.019	0.243
					0.506	1.008	0.242
							0.239
11	μBondapak C_{18}	1.40	5.0	3.5/6.5	0.484	0.924	0.256
					0.456	0.916	0.271
Range[d]					0.456–0.508	0.796–1.019	0.203–0.271
Mean[d]					0.479	0.940	0.238
CV, %[d]					2.93	6.56	6.75

[a] Did not meet the system suitability test.
[b] Rejected on basis of Dixon test (8) at the 95% confidence level.
[c] Associate Referee results.
[d] Does not include outlier from Collaborator 6 or results from Collaborator 4.

tract the ointment, an impurity from the hexane was extracted with the acetonitrile and produced a peak between the physostigmine peak and the internal standard peak in the HPLC chromatogram. This impurity was eliminated by switching to UV grade hexane.

Comments from Collaborators

All collaborators, except Collaborator 4, reported encountering no problems with the method. One collaborator reported that the HPLC method is a welcome improvement over the extraction and titration of the AOAC official method for physostigmine and its salts (3). Collaborator 3 noted that the physostigmine sulfate standard turned brown when it was dried overnight at 105°C. This drying time was much longer than called for in the method. One collaborator noted that the commercial preparations of physostigmine salicylate solution and physostigmine sulfate ointment had a slight coloration. Collaborator 4 had difficulty finding a suitable column and an instrument that functioned properly, and reported results that failed to meet the system suitability test. Collaborator 7 reported a small peak under the physostigmine peak that was not initially resolved in the physostigmine salicylate injection sample.

Collaborator 11 found 5 impurities in the physostigmine sulfate standard by increasing the concentration of standard injected in the proposed HPLC system; the major impurity was estimated to be 1.11% by area normalization. A physostigmine sulfate standard material currently under investigation as a USP Reference Standard gave the same pattern of impurities. This collaborator suggested that the resolution be increased to a minimum of 2.5 or 3.0.

Collaborator 11 also questioned the suitability of flurazepam as an internal standard because of the varying retention, with respect to physostigmine on different columns. The column that Collaborator 11 used was performing at less than

50% of initial efficiency but still met the system suitability test.

Several collaborators who tried using the Zorbax ODS column found it too retentive and not suitable.

Recommendations

Based on the collaborative results, it is recommended that the resolution factor be increased to a minimum of 3.0; that the drying time for physostigmine sulfate standard be changed to 2 h at 105°C; that UV grade hexane be specified; that the method as modified be adopted official first action for determining physostigmine, physostigmine salicylate, and physostigmine sulfate in drug preparations; that work continue to identify degradation products and their retention times.

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References

(1) Bently, K. W. (Ed.) (1966) *The Chemistry of Natural Products*, Vol. 1, *The Alkaloids*, 2nd Ed., Interscience Publishers, New York, NY, p. 158

(2) Hoover, J. E. (Ed.) (1970) *Remingtons Pharmaceutical Sciences*, 14th Ed., Mack Printing Co., Easton, PA., p. 905

(3) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, sec. **38.002 (a)** and **(b)**

(4) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD, pp. 624, 625

(5) Fletcher, G., & Davies, D. J. G. (1968) *J. Pharm. Pharmacol.* 20(Suppl.), 108S–113S

(6) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD, pp. 625, 626

(7) Kneczke, M. (1980) *J. Chromatogr.* **198**, 529–533

(8) Dixon, W. J. (1953) *Biometrics* **9**, 74–89

(9) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD, p. 625

Titrimetric Method With Ion Selective Electrode for Determination of Acetylenic Hypnotics

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An ion selective electrode has been used for indirect titrimetric assay of ethchlorvynol, ethinamate, and meparfynol carbamate. These acetylenic hypnotics form silver acetylide in the presence of excess silver. Excess silver is determined potentiometrically with standardized iodide solution and an iodide selective electrode. With this technique, 0.10-20 mg drug was determined with 95.7 ± 1.3-110.8 ± 5.9% accuracy. The procedure has been successfully applied to different pharmaceutical dosage forms.

Ethchlorvynol, ethinamate, and meparfynol carbamate are widely used as hypnotic and sedative drugs. These compounds are monosubstituted acetylene derivatives; the ethinyl group influences the drug activity (1). These compounds can be assayed by titrimetric (2-4), colorimetric (5, 6), spectrophotometric (7-10), nuclear magnetic resonance (11), and gas-liquid chromatographic methods (12-21).

Titrimetric methods depend on formation of silver acetylide, according to the equation:

$$R{-}C{\equiv}CH + nAgNO_3 \rightarrow R{-}C{\equiv}C.Ag(n-1)AgNO_3 + HNO_3$$

Liberated acid is titrated with standard alkali and the equivalent point is determined by methyl red-methylene blue mixed indicator (3, 4).

During the last few years, the scope of ion selective electrodes has been extended successfully for analysis of many pharmaceutical compounds (22). The technique has the advantages of rapidity and accuracy besides its sensitivity.

A useful application of the ion selective electrode is a microanalytical procedure for hypnotics; quantities as low as 100 μg can be assayed with good accuracy. In neutral medium, silver nitrate reacts with monosubstituted acetylenic compounds to form silver nitrate-silver acetylide complexes with various stoichiometric ratios (23, 24). These acetylides are hydrolyzed in acid medium, but in the presence of ammonia the reaction is straightforward.

$$R{-}C{\equiv}CH + Ag(NH_3)_2^+ + OH^- \rightarrow R{-}C{\equiv}C.Ag + H_2O + 2NH_3$$

The ratio of acetylenic compound to silver is 1:1; excess silver ions are determined potentiometrically with standard sodium or potassium iodide solution and the iodide selective electrode.

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METHOD

Apparatus and Reagents

(a) *pH meter.*—Orion Model 701 A digital pH/mV meter equipped with iodide electrode (Orion 94-53), and single junction reference electrode (Orion 90-01).

(b) *Water.*—Distilled, deionized, to prepare all solutions and standards.

(c) *Ionic strength adjustor (ISA).*—To keep constant background ionic strength: 2 mL 5M solution of reagent grade KNO_3 added to 100 mL standard iodide solution to bring background to 0.1M.

(d) *Standard solutions.*—(*1*) 0.1M sodium or potassium iodide solution: 15 g NaI or 16.6 g KI dissolved in 1 L water. (*2*) 0.1M silver amine: Accurately weigh 8.5 g silver nitrate (AR), dissolve in 100 mL water, add ammonia solution dropwise until precipitate redissolves, and dilute to 500 mL with water. Other concentrations are prepared by diluting these standards.

(e) *Drugs.*—Ethchlorvynol (BP grade): Abbott Laboratories Ltd, Queensborough, Kent, UK. Ethinamate (NF grade): CID Laboratories, Giza, Egypt. Meparfynol carbamate: Laténa Laboratories II, bis rue Bolzae, Paris 8, France. Each drug was prepared in 0.01M ethanolic solution to contain ca 10 μg-5 mg/mL.

Procedure for Pure Drug

Transfer 10 mL acetylenic compound (10 μg/mL) to 250 mL beaker, add 5-10 mL 1×10^{-3}M silver amine solution, and dilute to about 100 mL with water. Turn function switch to mV position, place electrodes in solution to ca 3 cm, and stir thoroughly; wait for stable millivolt reading, and record. Titrate with 0.5×10^{-3}M or 1×10^{-3}M sodium or potassium iodide solution. Plot mV readings against mL titrant to determine unreacted or excess silver ions. Determine end point from maximum of $\Delta E/\Delta V$. Each 1 mL of 1×10^{-3} silver nitrate is equivalent to 167.2 μg ethinamate, 144.6 μg ethchlorvynol, or 141.2 μg meparfynol carbamate.

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Table 1. Recovery of acetylenic hypnotics [a]

Amt taken, mg	Ethchlorvynol		Ethinamate		Meparfynol carbamate	
	Amt found, mg	Rec., %	Amt found, mg	Rec., %	Amt found, mg	Rec., %
0.03	0.01926	64.2 ± 13.9	0.03564	118.8 ± 14.5	0.01881	62.7 ± 27.2
0.05	0.04095	81.9 ± 8.3	0.04720	94.4 ± 9.6	0.0376	75.2 ± 8.1
0.10	0.1108	110.8 ± 5.9	0.1083	108.3 ± 8.3	0.0993	99.3 ± 10.9
0.12	0.13008	108.4 ± 1.5	0.12768	106.4 ± 8.0	0.1314	109.7 ± 6.8
0.15	0.1572	104.8 ± 2.4	0.1611	107.4 ± 6.4	0.1505	100.3 ± 5.4
0.30	0.3075	102.5 ± 3.5	0.3129	104.3 ± 1.2	0.3111	103.7 ± 4.4
0.50	0.5075	101.5 ± 3.3	0.5035	100.7 ± 1.2	0.50035	100.7 ± 3.2
1.0	1.025	102.5 ± 6.7	1.046	104.6 ± 2.2	1.037	103.7 ± 5.5
5	5.080	101.6 ± 5.8	5.085	101.7 ± 7.5	5.2	104.0 ± 1.6
10	10.34	103.4 ± 4.4	9.57	95.7 ± 1.3	9.97	99.7 ± 1.4
20	20.2	101.0 ± 4.2	19.74	98.7 ± 1.3	19.70	98.5 ± 2.8

[a] Mean (%) recovery for 3 determinations ± standard deviation.

Table 2. Comparison of reference and proposed methods for acetylenic hypnotics

Exp.	Ethchlorvynol, %		Ethinamate, %		Meparfynol carbamate, %	
	Proposed [a]	Official (3)	Proposed [a]	Official (4)	Proposed [a]	Official (3)
1	110.8	103.4	108.3	99.1	99.3	98.2
2	108.4	102.7	106.4	99.95	109.7	98.9
3	104.8	100.6	107.4	102.4	100.3	100.98
4	102.5	98.5	104.3	98.9	103.7	100.3
5	101.5	98.2	100.7	102.2	100.07	98.6
6	102.5	100.99	104.6	—	103.7	101.7
7	101.6	—	101.7	—	104.0	—
8	103.4	—	95.7	—	99.7	—
9	101.0	—	98.7	—	98.5	—
Mean	104.06	100.73	103.09	100.51	102.51	99.71
Variance	11.51	4.49	17.74	2.83	12.50	2.01
Student's *t*-test	2.129 (2.160) [b]		1.244 (2.179) [b]		1.502 (2.160) [b]	

[a] Mean (%) recovery for 3 determinations.
[b] The tabulated values of Student's *t*-test.

Repeat described procedure with 1–20 mg acetylenic compound, taking into consideration that the appropriate concentration of silver amine solution must be more than 3 times (6) the equivalent amount (to drive reaction to completion).

Procedure for Pharmaceutical Preparations

Quantitatively transfer aliquot of powdered tablets, content of capsules, or drops corresponding to 100 mg drug to 100 mL volumetric flask, add 50 mL ethanol, shake thoroughly, dilute with ethanol, and filter if necessary. Transfer 10 mL of this solution to another 100 mL volumetric flask and dilute to volume with ethanol. Transfer 1 mL to 250 mL beaker and proceed as above, beginning "... add 5–10 mL 1 × 10^{-3}M silver amine solution, ..."

Results and Discussion

Table 1 shows the percent recovery obtained with different amounts of pure compounds. Satisfactory results (average % recovery) were obtained with amounts more than 0.10 mg. Nonreproducible results were obtained with smaller quantities. This may be caused by the formation of silver acetylide–silver nitrate complex due to the large amounts of silver amine. However, more accurate and reproducible results were obtained for 0.1–20 mg drug.

Results in Table 1 were subjected to a paired comparison test (25); calculated values of Stu-

Table 3. Determination of acetylenic hypnotics in various dosage forms [a]

Preparation	Proposed procedure			Compendial method		
	No. of detn	Amt found, mg	Rec., %	No. of detn	Amt found, mg	Rec., %
Ethchlorvynol capsules, [b] 500 mg	4	497.25	99.45 ± 3.6	6	510.85	102.37 ± 1.63
Ethinamate tablets, [c] 500 mg	4	493.95	98.79 ± 2.14	5	497.35	99.47 ± 1.56
Meparfynol carbamate drops, [d] 20%	4	19288	96.44 ± 0.92	6	20016 (3)	100.08 ± 0.98
Meparfynol carbamate tablets, [d] 300 mg	4	301.5	100.5 ± 2.65	6	307.59 (3)	102.53 ± 1.09

[a] Mean (%) recovery ± standard deviation.
[b] Placidyl (Abbott).
[c] Valamid (Schering).
[d] N. Oblivon (Latéma).

dent's *t*-test for the results obtained using official methods are given in Table 2. Student's *t*-test shows no significance differences, although the coefficient of variation shows the proposed method to be less precise than the official methods. (Meparfynol carbamate is a nonofficial compound; the reference method is an adaptation of that for ethchlorvynol (3).)

This method was applied to the analysis of commercial preparations and recoveries were 96.44% ± 0.92 – 100.5% ± 2.65 (Table 3) compared with the official methods, which involved titration of not less than 100 mg of the drug as the liberated acid with standard alkali using mixed indicator (3, 4).

References

(1) Rutledge, T. F. (1968) *Acetylenic Compounds, Preparation and Substitution Reactions*, Reinhold Book Corp., New York, NY, pp. 314–333
(2) Miocque, M., & Gautier, J. A. (1958) *Bull. Soc. Chim. France* **5**, 467–469
(3) *British Pharmacopoeia* (1973) Pharmaceutical Press, London, UK, p. 191
(4) *U.S. Pharmacopeia XX* and *National Formulary XV* (1980) U.S. Pharmacopeial Convention, Inc., Rockville, MD, pp. 305–306
(5) Peter, H. (1973) *Clin. Chim. Acta* **43**, 139–141
(6) Rizk, M. S., Walash, M. I., & El-Brashy, A. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 88–90
(7) Kling, D. (1967) *Pharm. Ztg.* **112**, 1387–1390
(8) Peel, H. W., & Freimath, H. C. (1972) *J. Forensic Sci.* **17**, 688–692
(9) Wallace, J. E., Wilson, W. J., & Dahal, E. (1964) *J. Forensic Sci.* **9**, 342–352
(10) Wallace, J. E., Hamilton, H. E., Riloff, J. A., & Kenneth, B. (1974) *Clin. Chem.* **20**, 159–162
(11) Rueker, G., & Natrajan, P. N. (1967) *Arch. Pharm. Berlin* **300**, 276–281
(12) Robinson, D. W. (1968) *J. Pharm. Sci.* **57**, 185–186
(13) Davidson, A. W. (1970) *J. Assoc. Off. Anal. Chem.* **53**, 834–839
(14) Noirfalise, A. (1970) *J. Chromatogr.* **53**, 564–565
(15) Fricke, F. L. (1972) *J. Assoc. Off Anal. Chem.* **55**, 1162–1167
(16) Gibson, P. F., & Wright, N. (1972) *J. Pharm. Sci.* **61**, 169–171
(17) Evenson, M. A., & Poquette, M. A. (1974) *Clin. Chem.* **20**, 212–217
(18) Grove, J., & Martin, B. K. (1977) *J. Chromatogr.* **133**, 267–272
(19) Kleber, J. W., Bechtol, L. D., Branson, M. K., & Chernish, S. M. (1977) *J. Pharm. Sci.* **66**, 992–994
(20) Flannagan, R. J., & Lee, T. D. (1977) *J. Chromatogr.* **137**, 119–126
(21) McCurdy, H. H. (1977) *J. Anal. Toxicol.* **1**, 164–165
(22) Baiulescu, G. E., & Cosfret, V. V. (1977) *Applications of Ion Selective Membrane Electrodes in Organic Analysis*, John Wiley & Sons, Inc., New York, N.Y., p. 2
(23) Cheronis, M. D., & Ma, T. S. (1964) *Organic Functional Group Analysis*, Wiley-Interscience, New York, NY, p. 385
(24) Beverwijk, C. D. M., Van Der Kerk, G. J. M., Leusink, A. J., & Noltes, J. G. (1970) *Organometal. Chem. Rev. Sect.* **A5**(2), 215–280
(25) Hinchen, J. D. (1969) *Practical Statistics for Chemical Research*, Methuen, London, UK, p. 22

Spectrophotometric Determination of Menadione and Menadione Sodium Bisulfite

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A spectrophotometric method is described for the determination of menadione and menadione sodium bisulfite in bulk and in solution for injection. The method measures the intensity of the violet color (λmax 540 nm) developed when menadione reacts with thiosemicarbazide in alkaline medium. Beer's law is obeyed in the concentration range 4–40 μg/mL (r = 0.9995). The method is simple, sensitive, and particularly suited for routine analysis of official menadione sodium bisulfite injection. Results are comparable with the USP method.

Menadione and its sodium bisulfite derivative (MSB) are used as synthetic analogs of vitamin K. They are 3 times more active than natural vitamin K. Many analytical methods such as titrimetry (1–3), polarography (4, 5), fluorometry (6), gas chromatography (7, 8), and spectrophotometry (9–14) have been reported for determining the drug. The most widely used pharmacopeial method for determining menadione and MSB is titrimetry with ceric sulfate (1–3). There is a need for a more sensitive, simple, accurate, and time-saving method. Existing photometric methods either use difficult to obtain reagents (14), do not give reliable or reproducible results (12, 13), or are comparatively tedious, such as the USP XX assay for menadione injection. We report a new spectrophotometric assay for menadione and MSB based on the interaction of the drug with thiosemicarbazide to form an intense violet derivative. The method is simple, sensitive, and more specific for microgram levels of menadione and MSB. The procedure is also applied to the determination of MSB in some dosage forms.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Model 5886 (Prolabo), 10 mm path cells.

(b) *Thiosemicarbazide solution.*—Dissolve 0.5 g thiosemicarbazide in 10 mL water and dilute with n-propanol to 50 mL.

(c) *Standard menadione solution.*—Dissolve 10 mg pharmaceutical grade menadione in 100 mL n-propanol.

Preparation of Samples

Sample solution.—Dissolve weighed amount of menadione in n-propanol to yield solution containing 0.1 mg/mL. Treat MSB as described in USP to obtain menadione, which is used to prepare assay solution (0.1 mg/mL) in n-propanol.

K-Thrombyl injection.—Dilute 6 mL solution for injection to 20 mL with water and treat as described in USP to obtain menadione, which is used to prepare assay solution containing 0.1 mg/mL n-propanol.

Procedure

Pipet 1.0 mL assay solution into 5 mL volumetric flask. Add, in order, 0.2 mL thiosemicarbazide solution and 0.1 mL 0.1N NaOH. Mix well and let stand 30 min at 25°C. Dilute to volume with n-propanol and measure absorbance at 540 nm against blank prepared under same conditions with 1.0 mL n-propanol instead of sample.

Results and Discussion

A characteristic violet color with absorption maximum at 540 nm develops when menadione reacts with thiosemicarbazide in alkaline propanolic medium. Figure 1 shows the spectra of the reactants and products of the reaction in alkaline propanolic medium.

Optimum concentration of thiosemicarbazide for maximum intensity of color was 0.04% final dilution, which corresponds to 0.2 mL 1% thiosemicarbazide reagent/5 mL reaction mixture. Increasing concentration decreased the color intensity, probably due to the reducing effect of thiosemicarbazide on the chromogen formed.

For the reaction between menadione and thiosemicarbazide in alkaline medium, sodium hydroxide, ammonium hydroxide, sodium carbonate, sodium acetate, and ammonium acetate solutions were tried at different concentrations ranging from 0.05 to 1N. The color reaction occurs only in the presence of sodium hydroxide.

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Table 1. Quantitative determination of menadione and menadione sodium bisulfite

Sample	Claimed, mg	Recovery, % ($\bar{x} \pm$ SD)[a]	
		Proposed method	USP method
Pure menadione	20	101.5 ± 0.15	104.2 ± 1.14
Pure menadione sodium bisulfite	50	99.8 ± 0.23	98.3 ± 1.06
Menadione sodium bisulfite injection[b]	50	99.6 ± 0.43	96.8 ± 0.88

[a] Three determinations.
[b] K-Thrombyl injection (Roussel).

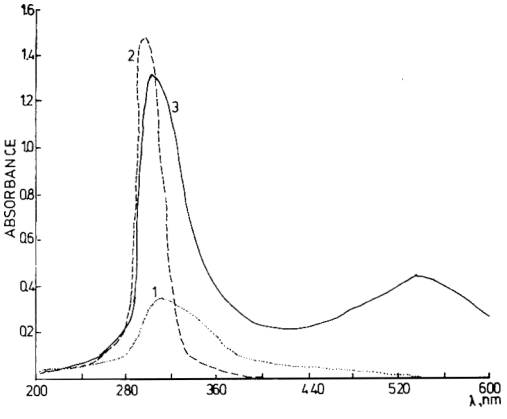


Figure 1. Spectra of alkaline *n*-propanol solution of 1, menadione (20 μg/mL); 2, thiosemicarbazide (0.4 mg/mL); and 3, chromogen (from 20 μg menadione/mL).

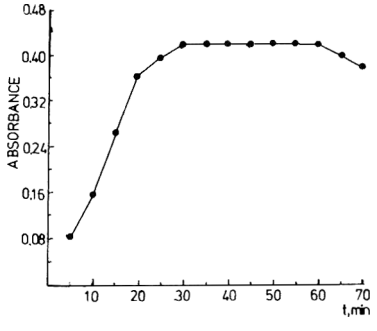


Figure 3. Effect of reaction time on color development.

Optimum concentration of sodium hydroxide for maximum intensity of color was 0.002N final dilution (corresponding to 0.1 mL 0.1N NaOH/5 mL reaction mixture). Higher concentrations did not significantly increase color intensity.

The solvent affects both the wavelength and intensity of maximum absorption. Water, methanol, ethanol, *n*-propanol, and isopropanol were studied. *n*-Butanol as well as higher organic solvents failed to mix with the aqueous reagents used. Figure 2 shows that *n*-propanol gave the highest intensity with a red shift. Maximum color intensity was obtained after 30 min at 25°C and was stable an additional 30 min (Figure 3).

A linear correlation (r = 0.9995) was found between absorbance at 540 nm and concentration of menadione in the range of 4–40 μg/mL. The apparent molar absorptivity was 3.6×10^3.

The continuous molar variation of menadione and thiosemicarbazide (Figure 4) showed that the interaction between these 2 compounds occurs on an equimolar basis. The mechanism of the

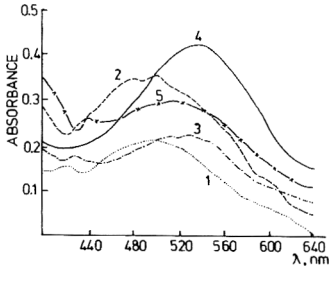


Figure 2. Absorption spectra of chromogen in 1, water; 2, methanol; 3, ethanol; 4, *n*-propanol; and 5, isopropanol.

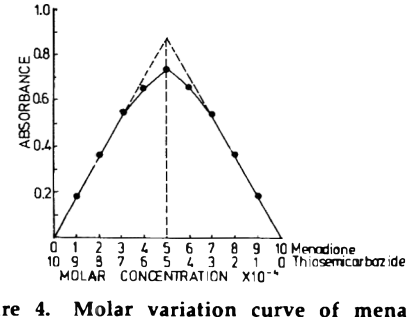
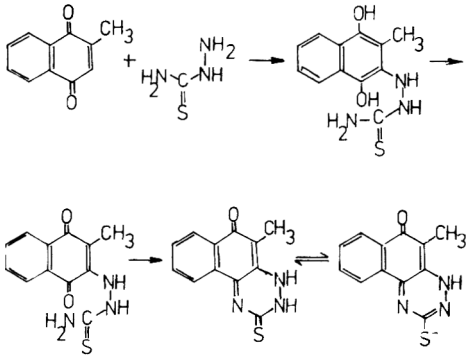


Figure 4. Molar variation curve of menadione thiosemicarbazide interaction.

reaction of naphthoquinones with thiosemicarbazide is not described in the available literature. Attempts to separate the chromogen in a pure form were unsuccessful due to the formation of a resinous, colored mass. The possibility of thiosemicarbazone formation is unlikely, because the reaction does not occur with anthraquinone or with MSB. Similar to reactions between amino or thiolated compounds and 1,4-naphthoquinone (15), a nucleophilic attack by thiosemicarbazide may occur according to the following scheme. The colored product forms



only in strong alkaline medium, as described here.

Application to Dosage Forms

The suggested method was applied to the quantitative determination of menadione and MSB in bulk and in injection. Comparison of the proposed method with the official USP method (3) showed the results to be comparable (Table 1).

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References

(1) *Egyptian Pharmacopeia* (1953) Elamiria Press, Cairo, Egypt, pp. 510, 511

(2) *European Pharmacopeia* (1969) Maisonneuve S. A., Paris, France, p. 325

(3) *U.S. Pharmacopeia* XX, and *National Formulary* XV (1980) U.S. Pharmacopeial Convention, Rockville, MD, pp. 472-474

(4) Mayo, A. M., & Nudelman, O. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 1460-1463

(5) Strokova, M. P., Feodrova, G. A., Shedov, B. D., & Saricheva, E. K. (1973) *Khim. Farm. Zh.* **7,** 53-55

(6) Veronse, F. M., & Meani, A. (1968) *Acta Vitaminol. Enzymol.* **22,** 303-308

(7) Winkler, V. W. (1973) *J. Assoc. Off. Anal. Chem.* **56,** 1277-1280

(8) Winkler, V. W., & Yoder, J. M. (1972) *J. Assoc. Off. Anal. Chem.* **55,** 1219-1220

(9) Martinson, E. E., & Meerovich, G. E. (1945) *Biokhimia* **10,** 258-260

(10) Canady, W. J., & Roe, J. H. (1956) *J. Biol. Chem.* **220,** 563-565

(11) Johnson, D. P., Critchfield, F. E., & Ruch, J. E. (1962) *Anal. Chem.* **34,** 1389-1390

(12) Pradhan, T. K. (1968) *J. Inst. Chemists (India)* **40,** 144-145

(13) Strohecker, R., & Henning, H. M. (1966) *Vitamin Assay Tested Methods,* Verlag. Chemie, Weinheim, p. 134

(14) Patel, J. C., Mehta, R. C., & Shastri, M. R. (1975) *Indian J. Pharm.* **37,** 141-143

(15) Patai, S. (Ed.) (1974) *The Chemistry of Quininoid Compounds,* Part 2, Wiley-Interscience, London, UK, pp. 881-913

Spectrophotometric Determination of Oxazepam and Dipyridamole in Two-Component Mixtures

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The Δp_j method is applied to simultaneous determination of oxazepam and dipyridamole in 2-component mixtures. Authentic mixtures and tablets containing oxazepam and dipyridamole were assayed by measuring the absorbances in 0.1N H_2SO_4 and 0.05M borax solutions and calculating the Δp_2 at 2 wavelength sets: 272-312 nm (at 8 nm intervals) and 288-328 nm (at 8 nm intervals). The concentration is evaluated by solving a pair of simultaneous equations. The results obtained are reasonably reproducible with a coefficient of variation less than 2%. The proposed method is compared with the ΔA modification of Vierordt's method of 2-component analysis. The Δp_j method gave more accurate and precise results.

Absorption spectrophotometry in the UV or visible range is well established as a rapid and reliable determinative technique for analyzing drug products when interferences can be eliminated. Figure 1(a) shows that the drugs of interest here, oxazepam and dipyridamole, exhibit pH-dependent UV/visible spectra such that dipyridamole interferes with the quantitative measurement of oxazepam when both drugs are present and a single-wavelength direct procedure is tried. However, both drugs can be accurately and precisely determined by spectrophotometry without separation when a polynomial approach is used. This approach involves mathematical treatment of data points from UV/visible spectra (obtained in each of 2 solvents) for preparations of the marketed product and the individual drug standards. Commercial tablets containing a mixture of these 2 drugs have been assayed by the Δp_j method, saving analytical time and effort when compared with alternative methods that use a combination of direct spectrophotometry for dipyridamole and lengthy isolation steps (including hydrolysis) followed by reaction colorimetry for oxazepam.

In 1971, Abdine et al. (1) introduced a differential spectrophotometric method of analysis (the Δp_j method) that depends on differences in orthogonal function coefficients. The authors (1) found that the peaks of certain compounds may split into subsidiary peaks without any change in intensity by changing the pH in a suitable interval. The behavior of these compounds restricts the application of the ΔA method (2) and the ΔA modification of Vierordt's method of 2-component analysis (3) because ΔA may not fulfill the requirements specified for its successful application. In these circumstances, the Δp_j method offers a solution for the determination of such compounds in the presence of irrelevent absorption. Thus, the contribution of a pH-insensitive irrelevant absorption may be cancelled by means of

$$\Delta p_{ji} = [\alpha_{jia} C_X + p_{ji}(z)] - [\alpha_{jib} C_X + p_{ji}(z)] \quad (1)$$

and

$$C_X = \Delta p_{ji} / \Delta \alpha_{ji} \quad (2)$$

where p_j is the coefficient of the polynomial, P_j (4); α_j is p_j (1%, 1 cm), i.e., the coefficient for A (1%, 1 cm) of the pure compound, X; C_X is the concentration; $p_{ji}(z)$ denotes the contribution from irrelevant absorption; the subscripts i, a, and b denote the wavelength range and the 2 different solutions, respectively. Thus, by choosing P_j and also the sets of wavelengths i so that p_{ji} is optimum in one solvent and negligibly small in the other solvent, Δp_{ji} can be used to evaluate compound X. The method has been successfully applied for determination of some drugs (1, 5, 6).

In the present work, the Δp_j method has been extended to analyze a 2-component mixture. Thus, the concentration of a mixture of 2 compounds X and Y can be evaluated from a pair of simultaneous equations of the following form:

$$\Delta p_{j1} = C_X \Delta \alpha_{j1} + C_Y \Delta \beta_{j1} \quad (3)$$

$$\Delta p_{j2} = C_X \Delta \alpha_{j2} + C_Y \Delta \beta_{j2} \quad (4)$$

Subscripts 1 and 2 refer to wavelength ranges; Δp_j denotes the difference between p_j of the mixture in the 2 different pH media a and b; C_X and C_Y are the concentrations of the compounds X and Y; while $\Delta \alpha_j$ and $\Delta \beta_j$ are the Δp_j (1%, 1 cm) of the compounds X and Y, respectively.

To obtain precise estimates of concentration for the analysis of a binary mixture by the proposed method, the following requirements are

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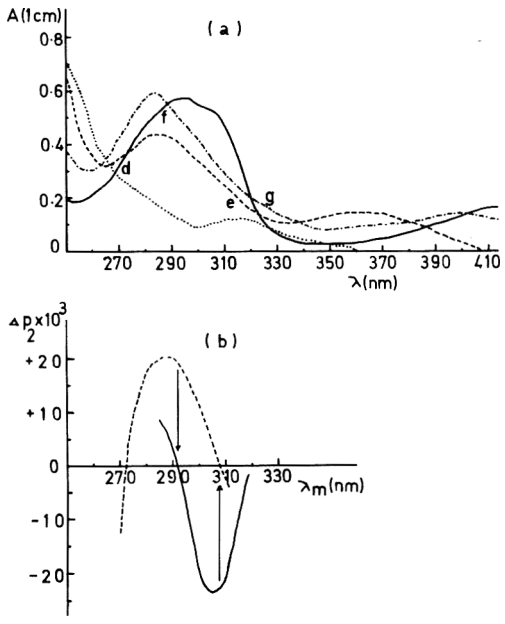

Figure 1. (a) Absorption spectra of 1.12 mg % oxazepam: (---) in 0.1N H_2SO_4, (·····) in 0.05M borax; 1 mg % dipyridamole: (-·-) in 0.1N H_2SO_4, (——) in 0.05M borax. (b) Δp_2 convoluted curves derived from absorption spectra: (---) oxazepam; (——) dipyridamole.

essential: (*1*) The Δp_j (1%, 1 cm) values for the components are sufficiently different at the chosen wavelength ranges to permit an accurate solution of Equations *3* & *4*, (*2*) preference for λ_{m1} and λ_{m2} (λ_m = the mean of the set of wavelengths) to correspond with a maximum or a minimum in the delta convoluted absorption curves of substances *X* and *Y* and taking in consideration that the value of $|\Delta p_j|.N_j^{1/2}$ for each component should exceed 140×10^{-3} for the coefficient of variation to be less than 1.0 (*1*).

Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Beckman Model 35.

(b) *Dipyridamole and oxazepam.*—Analytical grade (Karl Thomae GmbH, Biberach an der Riss, West Germany).

(c) *Reference drug solutions.*—Prepare solutions of 20 mg oxazepam and 50 mg dipyridamole in 100 mL ethanol. Dilute two 3 mL portions of each solution to 100 mL with 0.1N H_2SO_4 and 0.05M borax solutions, respectively.

(d) *Persumbran® tablets.*—Labeled to contain 10 mg oxazepam and 25 mg dipyridamole per tablet (Karl Thomae GmbH).

Procedure

Weigh and powder 20 tablets. Dissolve accurately weighed amount of powder equivalent to ca 20 mg oxazepam and 50 mg dipyridamole in 100 mL ethanol. Filter, and dilute two 3 mL portions of filtrate with 0.1N H_2SO_4 and 0.05M borax solutions, respectively. Measure absorbances of 1 cm pathlength of both solutions over wavelength range 272–328 nm at 8 nm intervals and at 283, 296, and 414 nm. Calculate coefficient p_2 for each solution at each wavelength range as follows:

$$p_{21} = (5A_{272} - A_{280} - 4A_{288} - 4A_{296} - A_{304} + 5A_{312})/84$$

$$p_{22} = (5A_{288} - A_{296} - 4A_{304} - 4A_{312} - A_{320} + 5A_{328})/84$$

Calculate α_{21}, α_{22}, β_{21}, and β_{22} in the same manner from absorbance measured for reference drug solutions. Calculate content of oxazepam and dipyridamole in tablets, according to Equations *3* and *4*.

Results and Discussion

Oxazepam exhibits a maximum absorption at 283 and 360 nm in 0.1N H_2SO_4 and at 315 nm in 0.05M borax solution. Dipyridamole exhibits a maximum absorption at 237, 283, and 400 nm in 0.1N H_2SO_4 and at 296 and 414 nm in 0.05M borax solution (Figure 1a). In 0.1N H_2SO_4, both oxazepam and dipyridamole have overlapping spectra in the region from 264 to 337 nm; in 0.05M borax, dipyridamole absorbs in the visible range in the region from 360 to 440 nm while oxazepam has a negligible absorption (Figure 1a).

According to general rules (*7*), the quadratic polynomial, P_2, was chosen because it makes a large contribution to segment de (Figure 1a) and a small contribution over the same segment in the absorption curves of oxazepam in 0.1N H_2SO_4 and 0.05M borax solutions, respectively; P_2 makes a large contribution to segment fg (Figure 1a) and small contributions over the same segment in the absorption curves of dipyridamole in 0.05M borax and 0.1N H_2SO_4 solutions, respectively.

Six-point orthogonal polynomials (*4*) were chosen as a compromise between the need for drawing maximal information from the continuous parts of the spectra and the laboriousness of the calculations. The optimum wavelength ranges λ_{m1} = 292 nm and λ_{m2} = 308 nm for oxazepam and dipyridamole, respectively, were

Table 1. Assay parameters for Δp_2 determination of oxazepam and dipyridamole in 2-component mixture

Compound	Concn, mg/100 mL	Wavelength range, nm	λ_m,nm	$\Delta p_2 \times 10^3$	$\lvert\Delta p_2\rvert.N_2^{1/2}.10^3$	Δp_2 (1%, 1 cm)
Oxazepam	1.12	272–312	292 (λ_{m1})	19.166	175.659	17.112 ($\Delta\alpha_{21}$)
		288–328	308 (λ_{m2})	0.571	—	0.510 ($\Delta\alpha_{22}$)
Dipyridamole	1.00	272–312	292 (λ_{m1})	−0.476	—	−0.476 ($\Delta\beta_{21}$)
		288–328	308 (λ_{m2})	−22.345	204.795	−22.345 ($\Delta\beta_{22}$)

chosen to correspond with a maximum or a minimum in the delta convoluted absorption curves (Figure 1b).

Table 1 shows the finally chosen assay parameters for the proposed method. Table 2 indicates that the 2 requirements (2) of the ΔA method are not fulfilled and the ΔA (1%, 1 cm) of oxazepam and dipyridamole at the chosen analytical wavelengths are not completely different.

Irrelevant absorption in spectra from tablets originates from the diluents (lactose, starch, sucrose), moistening agents (acacia mucilage, gelatin, liquid glucose), and lubricants (talc, stearic acid, and magnesium stearate). Different grades of these ingredients from different sources were dissolved in ethanol and filtered, and the spectra were measured in 0.1N H_2SO_4 and 0.05M borax solutions. The irrelevant absorption curves are not too complex (8) and are approximately linear over the wavelength range chosen. Furthermore, the irrelevant absorption is unaffected by the change in pH and grade-to-grade differences were negligible.

In general, an assay based on orthogonal functions rejects all components of the irrelevant absorption curve other than those which are used to calculate the assay coefficient (9). Furthermore, in the analysis of binary mixtures, the application of orthogonal functions eliminates linear irrelevant absorption which may originate from differences between batches of the components of the mixture and the reference sample used to establish the assay coefficients (9–11). In particular the Δp_j method is useful when the optimum conditions of the ΔA method are not fulfilled and the irrelevant absorption depends linearly on the spectra of the pure compounds (1).

Table 3 shows the results obtained for simultaneous determination of oxazepam and dipyridamole in authentic mixtures and purchased tablets (Persumbran tablets). The results obtained by the proposed method are both precise and accurate. The unsatisfactory results (Table 3) obtained by the ΔA modification of Vierordt's method of 2-component analysis (3) are due to the reasons mentioned previously (Table 2). In the analysis of the purchased tablets, the results obtained were compared with other spectrophotometric methods. Good agreement was obtained between the proposed method and the colorimetric method, i.e., measurements of the azo dye formed after hydrolysis of oxazepam and coupling with Bratton-Marshall reagent (12), and direct measurements of dipyridamole absorbance in 0.05M borax at 414 nm (Table 3).

Errors in the Δp_j method are mainly attributed to wavelength-setting errors which affect absorbances measured on steep slopes in the absorption curves (1). Furthermore, the error in an assay result based on the 2-component Δp_j method, which involves 4 coefficients calculated at 2 wavelength sets, would be greater than an error in a result based on 2 coefficients calculated at a single wavelength set in a one-component

Table 2. Fit of oxazepam and dipyridamole to requirements of ΔA method

Compound	Concn, mg/100 mL	λ_m[a] (nm)	A_a[b]	A_b[b]	$(A_a + A_b)$[c]	$\lvert(A_a - A_b)\rvert$[d]	ΔA (1%, 1 cm)
Oxazepam	1.12	283 (λ_1)	0.440	0.180	0.620	0.260	232
		296 (λ_2)	0.370	0.094	0.464	0.276	246
Dipyridamole	1.00	283 (λ_1)	0.582	0.510	1.092	0.072	72
		296 (λ_2)	0.463	0.561	1.024	0.098	98

[a] At λ_1 and λ_2, ΔA appears to be maximum for oxazepam and dipyridamole, respectively.
[b] A_a and A_b are the absorbances of 1 cm layers in 0.1N H_2SO_4 and 0.05M borax, respectively.
[c] Should not exceed 1.0.
[d] Should be about 0.430.

Table 3. Determination of oxazepam and dipyridamole in 2-component mixture[a]

Sample	Assays	Oxazepam, %			Dipyridamole, %		
		Δp_j method	ΔA method	Colorim. method (2)	Δp_j method	ΔA method	$A_{max.}$ method[b]
Authentic mixtures[c]	6	100.3 ± 1.2	97.3 ± 3.1	—	100.4 ± 1.8	102.2 ± 9.3	—
Tablets[d]	7	101.7 ± 1.4	107.3 ± 7.4	100.4 ± 1.1	98.0 ± 0.7	98.8 ± 4.1	98.2 ± 1.0

[a] Mean ± standard deviation.
[b] Absorbance measured at λ_{max} = 414 nm in 0.05M borax.
[c] Concentration range 0.6–1.1 mg/100 mL for oxazepam; 0.8–1.6 mg/100 mL for dipyridamole.
[d] Labeled to contain 10 mg oxazepam and 25 mg dipyridamole.

Δp_j method; therefore, great care must be taken in the choice of assay parameters.

Acknowledgments

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References

(1) Abdine, H., Wahbi, A. M., & Korany, M. A. (1972) *J. Pharm. Pharmacol.* **24,** 518–521
(2) Aulin-Erdtman, G. (1955) *Chem. Ind.* **74,** 581–582
(3) Wahbi, A. M., & Farghaly, A. M. (1970) *J. Pharm. Pharmacol.* **22,** 848–850
(4) Fischer, R. A., & Yates, F. (1953) *Statistical Tables for Biological Research*, 4th Ed., Oliver & Boyd, Edinburgh, UK, p. 80 et seq.
(5) Wahbi, A. M., Abdine, H., & Blaih, S. M. (1978) *Pharmazie* **33,** 96–98
(6) Amer, M. M., Ahmed, A. K. S., & Hassan, S. M. (1976) *J. Pharm. Pharmacol.* **29,** 291–293
(7) Wahbi, A. M. (1967) Ph.D. Thesis, University of London, UK
(8) Abdine, H., Wahbi, A. M., & Korany, M. A. (1971) *J. Pharm. Pharmacol.* **23,** 444–447
(9) Glenn, A. L. (1963) *J. Pharm. Pharmacol.* **15** (Suppl.), 123T–130T
(10) Abdine, H., Wahbi, A. M., & Korany, M. A. (1972) *J. Pharm. Pharmacol.* **24,** 522–524
(11) Korany, M. A. (1972) Dissertation, University of Alexandria, Egypt
(12) Walkenstein, S. S., Wiser, R., Gudmundsen, C. H., Kimmel, B., & Corradino, R. A. (1964) *J. Pharm. Sci.* **53,** 1181–1186

FOOD ADDITIVES

Atomic Absorption Spectroscopic Determination of Dimethylpolysiloxane in Juices and Beer

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A method is described for determining dimethylpolysiloxane (DMPS), an antifoaming agent, in juices and beer. The method involves adsorbing the beverage onto Florisil (an activated porous magnesium silicate), drying, and Soxhlet extraction with chloroform. The chloroform extract is evaporated to dryness and DMPS is determined in the residue after dissolution with methyl isobutyl ketone and aspiration into a nitrous oxide-acetylene flame of an atomic absorption spectrophotometer. The detection limit is about 0.2 ppm.

Dimethylpolysiloxane (DMPS) is used in antifoam preparations in the food industry. DMPS is soluble in most aliphatic and aromatic hydrocarbon solvents (1) but is difficult to extract quantitatively from aqueous samples by simple solvent extractions (2). Methods that have been reported for determining DMPS in liquid samples specify lengthy liquid-liquid solvent extractions (3) or azeotropic distillations with hazardous solvents such as benzene (4).

This paper describes a method that overcomes this extraction problem by adsorbing such samples onto a solid matrix (Florisil) and then, after drying, extracting the mixture for about 7 h with chloroform in a Soxhlet apparatus. The chloroform extract is evaporated to dryness, the residue is dissolved in methyl isobutyl ketone (MIBK), and DMPS is quantitated by atomic absorption spectroscopy (AAS), using a nitrous oxide–acetylene flame. The method is rapid and interference-free and results in good recoveries of DMPS. The atomic absorption quantitation is not specific for DMPS because all organic silicon compounds extracted by chloroform and soluble in MIBK are measured; however, these are not normally found in food, although traces could enter food from machinery treated with silicon lubricants.

METHOD

Use analytical grade reagents unless otherwise specified. Silicon grease or any oils containing silicon must not be used on apparatus at any stage of determination.

Reagents

(a) *Chloroform.*—Glass distilled, or equivalent.

(b) *Florisil.*—100–200 mesh.

(c) *Dimethylpolysiloxane standard solutions.*—(1) *Stock solution.*—1000 μg DMPS (DC 200 fluid, 20 centistokes, Dow Corning)/mL MIBK. (2) *Working solutions.*—Pipet 1.0, 10.0, and 20.0 mL portions of stock solution into separate 100 mL volumetric flasks and dilute to volume with MIBK to give 10, 100, and 200 μg DMPS/mL, respectively.

Apparatus

(a) *Soxhlet apparatus.*—Quickfit No. EX5/55/100, with 28 × 100 mm (Whatman) extraction thimble, 250 mL Quickfit flask with B34 socket, and Quickfit water condenser to fit B34 socket, or equivalent apparatus.

(b) *Atomic absorption spectrophotometer.*—Varian Techtron Model AA-5 equipped with IM-6 digital indicating module, BC-6 background corrector, Varian Si hollow cathode lamp operated at 10 mA and Varian hydrogen hollow cathode lamp, AB50 high temperature burner, and M80 automatic gas control unit. A fuel-lean nitrous oxide–acetylene flame was used (the red inner portion of the flame was ca 5 mm high) and burner height was adjusted to give maximum absorbance with 100 μg DMPS/mL standard (base of burner to top of burner was ca 53 mm). Spectrophotometer was set at 251.6 nm with slit width of 100 μm.

Determination

Add sufficient Florisil to almost fill a thimble (usually ca 23–24 g) to 200 mL beaker, pour 25 mL sample of well shaken juice or degassed beer onto Florisil, and mix thoroughly with glass rod. Place beaker containing adsorbed sample and glass rod into ca 60°C forced air oven to dry sample (ca 2 h). Remove sample from oven and

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Table 1. Dimethylpolysiloxane content of juice samples

Sample	No. of samples	Dimethylpolysiloxane, ppm
Orange juice	10	<0.2
	7	0.5, 0.7, 0.9, 1.5, 1.6, 1.6, 1.8
	4	9.4, 20, 20, 30
Orange juice conc.	5	<0.2
	2	1.5, 2.1
Various juices	3	<0.2
	6	0.6, 1.3, 3.2, 3.3, 13.5, 24.7
Fruit juice drinks	29	<0.2
	18	0.2–3.0
	7	4.3, 6.8, 8.6, 9.2, 9.5, 11.0, 13.5
	7	14.3, 20.5, 21.8, 22.8, 23.6, 51, 152

stir with glass rod to break up any lumps. Transfer sample from beaker to extraction thimble, wipe glass rod with tissue and plug thimble with same tissue, and extract 6–7 h with 150 mL $CHCl_3$ in Soxhlet apparatus. Disconnect 250 mL flask containing $CHCl_3$ extract from Soxhlet apparatus and evaporate to dryness on boiling water bath. Pipet 5.0 mL MIBK into same flask, rotating flask while pipetting so that MIBK washes sides of flask. Warm flask containing MIBK solution on water bath ca 1 min (do not evaporate any MIBK at this stage) to dissolve any extracted DMPS and then transfer contents to 10 mL glass-stopper test tube. Then aspirate MIBK solution directly into nitrous oxide–acetylene flame for determination of DMPS, using conditions specified under *Apparatus*, after zeroing instrument by aspirating pure MIBK into flame. Obtain calibration curve with standard solutions containing 10, 100, and 200 μg DMPS/mL MIBK. Read concentration of sample solutions from curve, divide this value by sample weight, and multiply result by 5 to give DMPS content of sample (ppm).

Results and Discussion

Ninety-eight samples including 21 orange juices, 7 orange juice concentrates, 9 other juices including apple, pineapple, apricot, tomato, lemon, and grapefruit juices, and 61 fruit juice drinks (combinations of some of the following ingredients—pineapple, mango, apricot, orange, and lemon fruit juices, and water, flavorings, and sugar) were analyzed for DMPS by this method. The results on these samples are summarized in Table 1. The recommended limit for DMPS in foods is usually 10 ppm; 14 samples exceeded the 10 ppm level and 1 sample had a high level of 152 ppm. An orange juice sample which had a DMPS content of 30 ppm was checked in triplicate and gave results of 28, 29, and 30 ppm. Eight samples of beer were also analyzed for DMPS and all were below the detection limit of 0.2 ppm.

The recovery of DMPS added to juices and beer was determined by spiking 25 mL samples with 62.5, 125, and 250 μg DMPS from a 500 μg DMPS/mL MIBK solution: 125, 250, and 500 μL quantities, respectively, of this solution were transferred from a 250 μL syringe. The spiked samples were treated the same way as samples. The recovery of DMPS at the 3 spiking levels (2.5, 5.0, and 10.0 ppm) was usually close to 100% (Table 2). Recoveries were also determined on orange juice spiked with antifoam AF emulsion (Dow Corning), which is an aqueous suspension containing about 30% DMPS (actual DMPS content was 30.7% as determined by dissolving 0.1150 g antifoam AF in 200 mL MIBK, aspirating this directly into the AAS system, and obtaining the DMPS content from the calibration curve). This form of DMPS is often used for beverages in the food industry. The antifoam AF was directly added to measured quantities of juice (500–3000 mL) at 6.8 and 27 ppm levels. The juice and added antifoam were magnetically stirred for about 5 h to ensure thorough mixing of antifoam, juice, and fruit pulp. The spiked juices were left to equilibrate in a refrigerator (<4°C) for 3 days and, after ½ h magnetic stirring, were analyzed for DMPS in the usual way. Recoveries were 100 and 95% at the 6.8 and 27 ppm spiking levels, respectively (Table 2).

The addition of DMPS in the form of antifoam AF more closely simulates the way DMPS is added to beverages during manufacture. One determination was also made on the pulp-free portion of the sample, spiked at the 27 ppm level, to discover whether DMPS is significantly trapped by the fruit pulp portion of juices. The bulk of the pulp was removed from this sample by centrifuging 5 min at 2000 rpm. The DMPS content of the pulp-free portion was 8.8 ppm, which means that 67% of the DMPS was retained

Table 2. Recovery of dimethylpolysiloxane added to juices and beer

Sample	No. of detns	Dimethylpolysiloxane, ppm			Av. rec., %	CV, %
		Unspiked	Added	Found		
Orange juice	5	<0.2	2.5	2.2, 2.4, 2.4, 2.4, 2.6	96	5.9
Orange juice	5	<0.2	5.0	4.7, 4.9, 4.9, 4.9, 5.1	98	2.9
Orange juice	5	<0.2	10.0	8.6, 8.6, 8.6, 9.4, 11.1	93	11.7
Apple juice	2	<0.2	10.0	8.6, 12.4	105	—
Pineapple juice	5	<0.2	10.0	8.9, 9.6, 9.9, 10.1, 10.3	98	5.6
Apricot juice	5	<0.2	10.0	8.8, 9.2, 9.3, 9.3, 9.5	92	2.8
Tomato juice	4	<0.2	10.0	9.6, 9.9, 10.1, 10.3	100	3.0
Mango juice drink	5	4.9	10.0	14.4, 14.4, 14.6, 14.6, 15.0	97	1.7
Beer	2	<0.2	2.5	2.0, 2.5	90	—
Beer	2	<0.2	5.0	4.2, 4.5	87	—
Beer	2	<0.2	10.0	9.0, 9.6	93	—
Orange juice[a]	5	<0.2	6.8	6.6, 6.7, 6.8, 6.8, 7.0	100	2.2
Orange juice[a]	9	<0.2	27.1	24.9, 24.9, 25.3, 25.9, 26.0 25.6, 26.2, 26.4, 26.9	95	2.6

[a] Samples spiked with antifoam AF instead of straight DMPS.

in the pulp; this could explain why simple solvent extractions from aqueous samples are not quantitative for DMPS (2).

The method is quite sensitive and a typical absorbance reading for a 10 μg DMPS/mL standard is 0.018 absorbance unit, equivalent to 2 ppm DMPS in a 25 mL sample. The detection limit, 0.2 ppm, was determined as the concentration of DMPS giving an absorbance reading equal to twice the standard deviation of 10 consecutive readings of the blank, which was 0.001.

The calibration curve is linear to at least 200 μg DMPS/mL concentrations, and results for a typical calibration curve are 0.018, 0.178, and 0.355 absorbance unit for 10, 100, and 200 μg DMPS/mL standards, respectively. The coefficients of variation of the various spikings (Table 2) are ≤3% in all but 3 cases. Because about 67% of the DMPS is adsorbed on the pulp, samples must be thoroughly mixed to ensure homogeneity, otherwise precision and accuracy of analyses can be seriously affected.

The method is interference-free and is not influenced by the presence of inorganic forms of silicon (5). The method (excluding the Soxhlet extraction) is rapid and does not require a great deal of operator skill; the actual handling time and number of steps per sample are minimal. The precision and sensitivity of the method are good and, because there is no appreciable residue from the chloroform extract of these samples, it is reasonable to expect that the detection limit could be lowered even further by simply using larger sample sizes (with proportional increases in the amount of Florisil adsorbent, larger extraction thimbles and apparatus); this could not be attempted in the present study because the necessary apparatus was not available.

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References

(1) Food Chemicals Codex (1972) 2nd Ed., National Academy of Sciences, Washington, DC, p. 255
(2) Kacprzak, J. L. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1142-1144
(3) Sinclair, A., & Hallam, T. R. (1971) *Analyst* **96,** 149-154
(4) Horner, H. J., Weiler, J. E., & Angelotti, N. C. (1960) *Anal. Chem.* **32,** 858-861
(5) Neal, P. (1969) *J. Assoc. Off. Anal. Chem.* **52,** 875-876

Rapid Dry Column Method for Determination of *N*-Nitrosopyrrolidine in Fried Bacon

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A rapid method has been developed for the determination of *N*-nitrosopyrrolidine (NPYR) in fried bacon at less than the 1 ppb level. Ground fried bacon is mixed with anhydrous sodium sulfate and Celite by using a glass mortar and pestle. This dry mixture is then added to a chromatographic column containing a layer of acid-Celite. The column is washed with pentane-dichloromethane, and the nitrosamines are eluted with pure dichloromethane. The eluate is concentrated, and the nitrosamines are quantitated by using a gas chromatograph interfaced with a thermal energy analyzer. Recovery of the nitrosamine internal standard, *N*-nitrosoazetidine, added at the 10 ppb level, was over 90%. The results obtained by this method are in good agreement with the mineral oil distillation procedure currently used in the FSIS monitoring program. Because 25 samples can be analyzed per day per person, this simple screening procedure offers advantages over other methods.

In 1978, the USDA Food Safety and Quality Service (now Food Safety and Inspection Service (FSIS)) established a 10 ppb violative level for volatile nitrosamines in fried, cure-pumped bacon (1) because this product consistently contained *N*-nitrosopyrrolidine (NPYR) and, to a lesser extent, *N*-nitrosodimethylamine (NDMA). Since then, the mineral oil distillation–gas chromatographic (GC) thermal energy analyzer (TEA) screening method developed by Fine et al. (2) has been the most widely used procedure for determining volatile nitrosamines in cured meat products. Their method involves vacuum-distillation of the nitrosamines from a mixture of comminuted sample, mineral oil, and a small amount of base, followed by extraction of the aqueous distillate with dichloromethane (DCM), and concentration of the DCM before detection and quantitation. This procedure is used by FSIS in their monitoring program. Samples presumably in violation are then analyzed by the more broadly applicable and lengthy FDA multidetection procedure, which involves sample digestion in methanolic potassium hydroxide, liquid-liquid extraction of the nitrosamines into DCM, distillation from base, acidification of the aqueous distillate, extraction with DCM, concentration before detection by GLC with alkali flame ionization detection (AFID), and a column chromatographic cleanup step for subsequent GLC/mass spectrometric confirmation (3). In the original multidetection method, the nitrosamines were detected by GLC, using a modified thermionic or alkali flame ionization detector (4). Havery et al. (3) analyzed 18 fried bacon samples and generally found good agreement between NPYR values obtained by the GLC/AFID, GLC/TEA, and mineral oil distillation-GLC/TEA procedures. Greenfield et al. (5) recently carried out a 9-laboratory collaborative study on fried bacon fortified with 6 volatile nitrosamines at 6 levels ranging from 0 to 17 ppb and 10 ppb *N*-nitrosodipropylamine internal standard, using the mineral oil distillation-GLC/TEA procedure.

However, the mineral oil distillation-GLC/TEA and other published procedures (6) have several disadvantages. The most important of these is the lengthy analytical time involved. A more rapid method therefore is needed for the routine determination of volatile nitrosamines in fried bacon. We have developed a rapid dry column method based on the principle employed by Maxwell et al. (7) for isolating lipids from muscle and adipose tissue.

METHOD

Note: Nitrosamines are potential carcinogens. Exercise care in handling these materials.

Reagents

(a) *Celite 545.*—Not acid-washed (Fisher Scientific Co.). Run reagent blank before start of sample analysis, particularly if new bottle of Celite is used. If interfering chromatographic products are noted, prewash Celite twice with dichloromethane, then dry 4 h in 120°C vacuum oven before use.

(b) *Dichloromethane (DCM) and n-pentane.*—

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Distilled in glass (Burdick & Jackson Laboratories, Inc.).

(c) *Hydrochloric acid.*—6N. Extract once with equal volume of DCM to remove impurities.

(d) *Sodium sulfate.*—Anhydrous, granular (Mallinckrodt No. 8024).

(e) *Internal standard solution.*—0.10 μg *N*-nitrosoazetidine (NAZET)/mL DCM.

(f) *N-Nitrosopyrrolidine (NPYR) and NAZET GLC working standard.*—Each 0.10 μg/mL DCM.

Apparatus

Usual laboratory equipment and the following items:

(a) *Mortar and pestle.*—Glass, 473 mL (16 oz), A. H. Thomas Co.

(b) *Chromatographic column.*—Glass, 350 × 32 mm id with 60 × 6 mm id drip tip.

(c) *Evaporative concentrator.*—Kuderna-Danish (KD), 250 mL; concentrator tube, 4 mL, graduated; Snyder (3-section) and micro Snyder distilling columns (Kontes Glass Co.).

(d) *Tamping rod.*—Glass, 450 mm long with 12 mm diam. disc prepared by glassblower.

(e) *Gas chromatograph-thermal energy analyzer.*—Varian Aerograph gas chromatograph Model 2700, or equivalent, interfaced with a thermal energy analyzer Model 502. Operating conditions: 2.7 m × 3.2 mm stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas-Chrom P; helium carrier gas 35 mL/min; column 180°C isothermal, injector 200°C, TEA furnace, 450°C; TEA vacuum 1.5 mm; liquid nitrogen–ethanol cold trap.

Procedure

(a) *Sample analysis.*—Weigh 10 g Celite into 250 mL beaker. Add 10 mL 6N HCl,[2] ca 3 mL at a time, and stir Celite with small glass rod until mixture is fluffy and uniform in texture. Using a powder funnel, pour acid-Celite into chromatographic column containing glass wool plug at bottom. Insert tamping rod through Celite and tamp from bottom up to achieve height of ca 25 mm. Accurately weigh 10.0 ± 0.1 g doubly ground fried bacon and quantitatively transfer sample to mortar. Add 1.0 mL internal standard solution (equivalent to 10 ppb) to bacon sample, using 1.0 mL transfer pipet. Then add 25 g Na_2SO_4 and mix with pestle ca 30 s. Add 20 g Celite to mortar and grind 15–20 s until Celite is thoroughly mixed with Na_2SO_4 and bacon. Then, grind with moderate pressure for an additional 2 min. Quantitatively transfer free-flowing dry mixture into chromatographic column, and tamp with glass rod to achieve total height of ca 100 mm. Add 30 g Na_2SO_4 to top of column. Rinse mortar and pestle with 10 mL pentane–DCM (95 + 5), and add rinse to column, immediately followed by 90 mL of same solvent. Collect eluate in 100 mL graduated cylinder. When level of solvent in column drops so that it just touches top of Na_2SO_4, add 125 mL DCM at one time. After 85 mL of wash eluate has been collected, discard and change receivers. Collect remaining eluate in 250 mL KD flask equipped with 4 mL concentrator tube. (Some samples yield turbid effluent; this is normal.) When column stops dripping, remove KD flask, add 2 small boiling chips to flask, attach 3-section Snyder column, and concentrate eluate to 4 mL on steam bath. Continue concentration (add new boiling chip) to 1.0 mL with micro Snyder column in 70°C water bath. Note: Room temperature should be <24°C during analysis of sample.

(b) *Nitrosamine determination.*—Inject 9.0 μL GLC working standard at lowest attenuation that yields signal at least one-third full scale TEA response, and measure peak heights. Repeat to assure good reproducibility of retention time and response. Inject 9.0 μL concentrated nitrosamine-containing sample, measure response of the 2 nitrosamines, and calculate NPYR in ppb, using following formula:

$Z = YACV \times 1000/XBW$, where Z = μg NPYR/kg (ppb); V = total volume of sample = 1.0 mL; X = peak height of NPYR in standard; Y = peak height of NPYR in sample; C = concentration of standard = 0.10 μg/mL; A = μL of standard injected; B = μL of sample injected; W = weight of sample analyzed = 10.0 ± 0.1 g.

Statistical Analysis

One-tailed paired *t*-tests or analyses of variance were performed on the measured nitrosamine according to methods described by Snedecor and Cochran (8). Where only the statistical summary is presented, the raw data are available on request. The uncorrected NPYR data were reported as measured and the corrected NPYR data were adjusted for the recovery of the internal nitrosamine standard. For statistical purposes, NPYR data were reported to 2 decimal places.

Results and Discussion

The recovery of 14 volatile nitrosamines added to nitrosamine-free fried bacon was determined

[2] Author's note: Phosphoric acid (6N) has replaced hydrochloric acid.

Table 1. Recovery of volatile nitrosamines at the 10 ppb level from nitrosamine-free bacon

N-Nitroso compound	Rec., % Range	Mean (n = 4)	SD	CV, %
Dimethylamine	100.0–104.0	101.40	1.89	1.86
Methylethylamine	87.7–94.7	91.43	3.75	4.10
Diethylamine	69.7–77.3	74.20	3.38	4.55
Methylpropylamine	42.7–62.7	52.28	8.23	15.75
Ethylpropylamine	NR[a]	—	—	—
Dipropylamine	NR	—	—	—
Ethylbutylamine	NR	—	—	—
Propylbutylamine	NR	—	—	—
Methylamylamine	NR	—	—	—
Azetidine	90.7–95.7	93.20	2.17	2.33
Dibutylamine	NR	—	—	—
Piperidine	62.5–74.1	68.08	4.77	7.01
Pyrrolidine	103.6–110.0	105.43	3.57	3.38
Morpholine	94.1–104.1	98.08	4.32	4.41

[a] NR = no recovery.

Table 2. Statistical analysis on repeatability of ERRC method

		NPYR, uncorrected			NPYR, corrected			% Recovery of NAZET		
Sources of variation	df	SS	MS	F	SS	MS	F	SS	MS	F
N-Nitrosopyrrolidine	10	463.10	46.31		472.53	47.25		1607.61	160.76	
Error	23	3.09	0.13	344.77**	3.03	0.13	359.16**	404.47	17.59	9.14**
Total	33	466.19	—		475.54	—		2012.09	—	
Repeatability[a]			0.37 ppb			0.36 ppb			4.19%	

** P<0.01.
[a] Repeatability = $\sqrt{MS_{error}}$.

to assess the applicability of our procedure, referred to here as the ERRC method (Table 1). The 2 nitrosamines that commonly occur in fried bacon, NDMA and NPYR, as well as *N*-nitrosomethylethylamine, -azetidine, -morpholine, were recovered at a mean level >90%. The mean recovery of the other nitrosamines varied from 0 to 74%. The mean recovery for *N*-nitrosopiperidine, which is occasionally found in fried bacon, was 68%. Because *N*-nitrosodipropylamine (NDPA), the internal standard in the mineral oil method, was not recovered, NAZET was selected as the internal standard. The possibility of this 4-membered heterocyclic nitrosamine being naturally present or formed in food products was considered unlikely. Also, preliminary evidence indicates a highly significant correlation (r^2 = 0.925, P <0.01) between NAZET and NPYR recoveries. Although the ERRC method is applicable to all the volatile nitrosamines detected in fried bacon, this study was restricted to the determination of NPYR, because it is found in the highest concentration and is most likely to exceed the violative level.

Eleven bacon samples were analyzed, 6 in quadruplicate and 5 in duplicate, to determine within-laboratory repeatability of the ERRC

Table 3. Determination of *N*-nitrosopyrrolidine (ppb) in fried bacon by the ERRC and mineral oil-TEA methods

	Mineral oil			ERRC		
Sample No.[a]	Uncorr.	Corr.	NDPA, % rec.	Uncorr.	Corr.	NAZET, % rec.
1	5.28	6.29	84.0	4.64	5.29	87.7
	5.45	5.85	93.2	4.40	5.13	85.8
2	17.75	22.05	80.5	16.46	21.57	76.3
	14.84	20.14	73.7	15.82	19.36	81.7
3	6.16	6.88	89.5	5.29	6.71	78.9
	6.58	6.38	103.2	5.43	6.52	83.3
4	6.33	6.01	105.4	4.13	4.64	89.1
	4.94	5.26	93.9	4.19	4.83	86.8
5	15.14	15.92	95.1	16.23	16.70	97.2
	13.87	14.47	95.8	15.25	16.93	90.1
6	7.14	7.01	101.8	6.85	7.42	92.3
	6.08	6.31	96.3	6.71	7.10	94.5
7	13.16	12.70	103.6	13.08	13.57	96.4
	13.61	13.24	102.8	13.69	13.69	100.0
8	5.71	5.50	103.9	5.22	6.40	81.6
	5.84	5.62	103.9	5.37	5.91	90.8

[a] Duplicate determinations.

Table 4. Statistical analysis on determination of *N*-nitrosopyrrolidine in fried bacon by the ERRC and mineral oil-TEA methods

Determination	No. of detns	Mean ($\bar{X}$) Min. oil ($\bar{x}_1$)	Mean ($\bar{X}$) ERRC ($\bar{x}_2$)	SD between samples Min. oil (s_1)	SD between samples ERRC (s_2)	Results between methods $\bar{x}_1 - \bar{x}_2$	Results between methods $s_1 - s_2$	Min. oil vs ERRC t
NPYR, uncorr. (ppb)	16	9.25	8.92	4.63	5.22	0.33	−0.59	0.342
NPYR, corr. (ppb)	16	9.98	10.11	5.80	6.02	−0.13	−0.22	−0.211
% Rec. of int. std[a]	16	95.40	88.38	8.83	6.53	7.12	2.30	1.532

[a] NAZET for ERRC method, NDPA for mineral oil method.

method. Determinations of NPYR ranged from 2.23 to 16.93 ppb, corrected (1.78 to 16.23 ppb, uncorrected) and recovery of the NAZET internal standard ranged from 73.7 to 101.8% with a mean of 91.3%. Analysis of variance of the results (Table 2) indicated that repeatability of NPYR determination is 0.36 ppb (0.37 uncorrected) and standard deviation of recovery of NAZET standard is 4.19%.

A ruggedness test of the ERRC method for the determination of 1.5 and 6.0 ppb NPYR, conducted by using different grinding, packing, and solvent elution steps of the procedure specified in the experimental section, indicated that results were not significantly different. However, the column-packing step of the procedure did lead to significant differences in the determinations of 1.5 ppb NPYR. When columns were packed too tightly, determinations varied 16% (23%, uncorrected) at 1.5 ppb NPYR compared with 0.3% (1.0%, uncorrected) at 6.0 ppb NPYR.

Comparative analysis of the ERRC and mineral oil methods was obtained with duplicate determinations of 8 samples of fried bacon containing from 4 to 20 ppb NPYR (Table 3). The 2 methods require a different internal standard because NDPA which is used in the mineral oil procedure, is not recoverable by this procedure, and NAZET, which is used in this procedure, sometimes decomposes during the distillation step of the mineral oil procedure. Statistical analysis of these determinations indicated that the results were equivalent (Table 4). Means of NPYR determinations with this method were 1.3% higher (3.6% lower, uncorrected) and standard deviation of the determinations was 3.7% higher (11.3%, uncorrected) than with the mineral oil reference method. Recovery of internal standard averaged 7.5% lower and varied 35% less than with the reference method. From the mean differences and standard deviations of determinations and recovery of internal standard, a t-test ($P = 0.05$) indicated that results with the 2 methods were not significantly different.

Determination of NPYR tends to be higher with the mineral oil method than with the multidetection method (3, and unpublished data). We therefore undertook a study to determine if NPYR was produced as an artifact during analysis. From 0 to 100 ppm sodium nitrite ($NaNO_2$) was added to nitrite-free bacon, and then determined by both the ERRC method and the mineral oil procedure. With increasing levels of $NaNO_2$, NPYR increased in the mineral oil procedure; up to 4 ppb was found when 50 ppm sodium nitrite was added (Table 5). No NPYR was detected when the samples were analyzed by the ERRC method. High residual nitrite in fried bacon could result from undercooking, which may be caused by slice thickness or compositional factors that affect the rate of frying. This bacon, when analyzed by the mineral oil procedure could produce more NPYR as an artifact. Because of this possibility, several investigators now add sodium ascorbate and/or α-tocopherol before distillation to avoid artifactual nitrosamine formation in the mineral oil method (unpublished). This precaution is not necessary with the ERRC method.

A limited interlaboratory study using the ERRC method was conducted on fried bacon containing 0–50 ppb normally incurred NPYR. Samples were analyzed in 3 laboratories; how-

Table 5. Effect of added sodium nitrite on *N*-nitrosopyrrolidine formation, determined by the mineral oil and ERRC methods[a]

Sample No.	$NaNO_2$ added, ppm	NPYR, ppb Min. oil	NPYR, ppb ERRC
1	0	0.23	ND[b]
2	10	0.99	ND
3	25	1.34	ND
4	50	3.58	ND
5	100	4.84	ND

[a] Two separate experiments in duplicate.
[b] ND = none detected.

Table 6. Interlaboratory collaborative study on determination of *N*-nitrosopyrrolidine (ppb, corrected) in fried bacon by ERRC method

Sample No. (dupls)	Analyst 1		Analyst 1A		Analyst 2		Analyst 3	
	NAZET, % rec.	NPYR	NAZET, % rec.	NPYR	NAZET, % rec.	NPYR	NAZET, % rec.	NPYR
1	108.2	27.73	89.5	30.21	97.7	30.71	88.8	31.85
	103.4	27.61	91.7	31.07	74.1	33.32	84.9	29.20
2	102.3	10.76	89.5	10.39	103.5	8.75	82.5	11.33
	102.3	10.83	95.8	10.72	82.4	9.48	93.6	10.66
3	100.0	8.92	85.3	8.92	103.5	8.75	91.1	8.79
	100.0	9.08	91.6	8.80	78.3	8.39	82.2	8.65
4	108.0	N.D.	96.8	N.D.	92.0[a]	N.D.	92.3	N.D.
	109.1	N.D.	96.9	N.D.	91.8	N.D.	96.1	N.D.
5	100.0	48.82	98.9	45.57	100.0	47.01	92.2	49.12
	94.6	47.80	100.0	47.32	95.7	44.92	92.4	46.66
6	91.3	2.86	95.6	2.36	82.6	2.35	87.3	2.60
	90.3	2.61	92.8	2.61	85.7	2.70	85.9	2.76
7	86.8	7.90	89.0	8.23	91.3	7.36	82.9	7.91
	96.7	7.60	97.6	8.23	104.8	7.34	77.4	8.23
8	91.4	6.69	97.6	6.61	88.1	4.19	85.0	6.88
	98.9	6.18	95.2	6.10	100.0	4.92	83.3	6.60

[a] Average used for statistical purposes, no internal standard added.

ever, in Laboratory 1, 2 different analysts performed the assay. Because each analyst worked independently, the data were treated statistically as if from 2 separate laboratories. Corrected results of the study (Table 6) were treated statistically because the variation was less than in the uncorrected data. The average within-laboratory recoveries with standard deviation for the internal standard were: 1A, 99.0 ± 6.7%; 1B, 94.0 ± 4.2%; 2, 92.0 ± 9.5%; 3, 87.4 ± 5.2%. The analysis of variance on the corrected results is shown in Table 7. A significant (P <0.01) difference between the bacon samples was observed with an F-test as expected because fried bacon samples with a wide NPYR range were intentionally used. No significant laboratory effect nor laboratory × sample interaction was indicated by the analysis of variance. The standard deviations for reproducibility and repeatability, determined as prescribed by Steiner (9), were 1.03 and 0.71, respectively. This compares favorably with the values of 1.34 and 1.04 for reproducibility and repeatability obtained in the recent collaborative study (5) on the FSIS mineral oil procedure. A collaborative study of the ERRC method involving a larger number of laboratories is planned.

In conclusion, we are reporting a method for the determination of NPYR in fried bacon, which is rapid, less susceptible to artifactual nitrosamine formation, and quantitatively as good as the currently employed method. With the ERRC method, 25 or more samples per analyst per day can be analyzed with limited glassware, thereby significantly reducing the cost of analysis.

Table 7. Analysis of variance on interlaboratory collaborative study

Variation	df	SS	MS	F
Sample	7	14635.9	2090.8	4182.5**
Laboratory	3	3.9	1.3	<1
Lab × sample	21	35.1	1.7	3.3
Error	32	16.0	0.5	
Total	63	14690.9		

** P <0.01.

Acknowledgment

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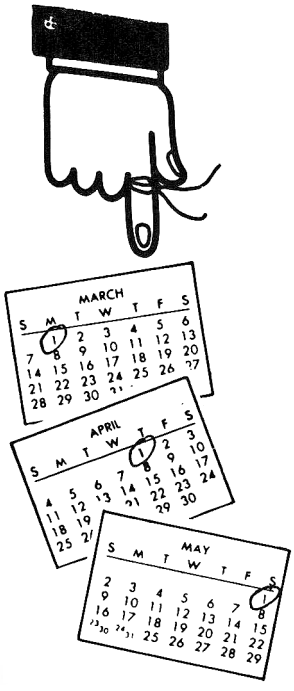
References

(1) *Fed. Regist.* (1978) **43**, 32136-32137
(2) Fine, D. H., Rounbehler, D. P., & Oettinger, P. E. (1975) *Anal. Chim. Acta* **78**, 383-389
(3) Havery, D. C., Fazio, T., & Howard, J. W. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1374-1378
(4) Fazio, T., White, R. H., Dusold, L. R., & Howard, J. W. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 919-921
(5) Greenfield, E. L., Vasco, G. A., Legette, L., & Malanoski, A. J. (1981) *J. Assoc. Off. Anal. Chem.* submitted for publication

(6) Egan, H., Preussmann, R., Castegnaro, M., Walker, E. A., & Davis, W. (1978) *Environmental Carcinogens Selected Methods of Analysis*, Vol. 1, *Analysis of Volatile Nitrosamines in Food*, IARC Scientific Publ. No. 18, International Agency for Research on Cancer, Lyon, France
(7) Maxwell, R. J., Marmer, W. N., Zubillaga, M. P., & Dalickas, G. A. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 600–603
(8) Snedecor, G. W., & Cochran, W. G. (1974) *Statistical Analysis*, 6th Ed., Iowa State University Press, Ames, IA
(9) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA



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Comparison of Nitrite and Nitrate Determinations in Vegetables: Suitability for Accurate and Automated Measurements

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A method is reported for improved accuracy for nitrate and nitrite determinations in aqueous solutions, especially plant extracts. The sampling procedure can be adapted for automated determinations: At least 6 aliquots with increasing volume of nitrate-containing solutions are subjected to the reducing agent under stabilized conditions to obtain nitrite, treated with one of 2 different diazotization reagents, and measured colorimetrically. The slope of the curve, absorbance vs sampled volume, is determined by linear regression; this slope is directly dependent on the nitrate (nitrite) content. Original nitrite is determined by omitting the reducing step. Low nitrate levels (<0.1 μg/mL) can be determined directly with the sulfanilamide-*N*-(1-naphthyl)ethylenediamine dihydrochloride diazotization procedure or indirectly by constant nitrate addition, with the sulfanilic acid-1-naphthylamine hydrochloride diazotization mixture (Griess reagent). The latter procedure can be used to measure concentrations up to 20 μg/mL.

During the past 10 years, attention has been focused on the natural and occasional presence of nitrate and nitrite in foodstuffs, especially drinking water, fresh and frozen vegetables, and canned foods. Factors contributing to concentrations of these almost non-intentional additives in food were reported previously (1-4).

Accurate nitrate and nitrite determinations are of interest because both nitrates and nitrites may act as precursors to the formation of carcinogenic nitrosamines in the body. The chemical method for nitrate determination in food is based on the reduction to nitrite by powdered cadmium at pH 9.6; the nitrites, original and reduced nitrates, can be determined by colorimetry after diazotization to water-soluble (5) or organic-soluble (6) colored products.

During our experiments, we observed that the extrapolated absorbance value for zero concentration of pure nitrate solutions differed from the extrapolated absorbance values for infinite dilution in each of the investigated food extracts (Table 1). This is probably due to adsorption of nitrite on the cadmium powder during the reduction stage, a phenomenon yet to be reported. This food-dependency through variable blanks may be an important source of error, especially when low nitrate concentrations are investigated and when constant blank values are used in automated nitrate determinations. A method designed to avoid variable blanks was briefly reported previously (4). The present report gives more details concerning the theoretical aspects and includes supporting data to prove the applicability of the proposed method. Two different diazotization mixtures were tested to determine their reproducibility, accuracy, and usefulness for measuring nitrate concentrations.

Experimental

Suitable food extracts were obtained as described previously (4). About 10 g fresh material or 1 g dried product was heated 30 min in a 250 mL beaker with 50 mL water on a water bath, and then homogenized 1 min with a Bamix mixer. After cooling, the plant-mixture was transferred into a 250 mL flask, deproteinized with Carrez solutions, and diluted to volume with water. The clear, nearly colorless filtrate was collected in a dry flask through a S & S No. 597½ filter paper.

This filtered solution was sampled 6 times by pipetting 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL into separate receiving tubes; 5 mL NH_4Cl-NH_4OH buffer solution (pH 9.6) was added to each tube. These solutions were diluted to 25 mL with water and then pressed under controlled circumstances through a Cd column (Figure 1). The treated solutions and rinsings (±40 mL) were collected and adjusted to 50 mL with water. To prepare a calibration curve, a sodium nitrate solution containing 10 μg nitrate nitrogen/mL (6.068 g $NaNO_3$/L diluted × 100) was sampled and treated similarly.

A 10 mL aliquot from each 50 mL solution was mixed in separate dry receiving tubes with one of 2 diazotization mixtures:

(*1*) 10 mL Griess mixture (4). A blue-red stable color is obtained in presence of nitrite after

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Table 1. Comparison of calculated and extrapolated values of nitrite for infinite dilution of different food extracts [a]

Product	g Sample/ 250 mL	NO_3^--N concn of ext, μg/mL	NO_3^--N concn of prod., μg/g	Extrapd zero value, *A*	Calcd slope, *A*/mL
Red cabbage	20.6539	1.82	22.03	0.039	0.0467
Cheese	4.3873	0.25_2	14.3	0.039	0.0064
Clover (chem. fertilizer)	4.5762	5.31_6	290	0.032	0.1363
Clover (biol. fertilizer)	4.9200	2.59	131	0.030	0.0664
Ham	24.5480	1.12_7	11.5	0.025	0.0289
$NaNO_3$	0.0151 7[b]	10.00	—	0.009	0.2565
H_2O blank	—	—	—	0.015	—
Cd blank	—	—	—	0.026	—
Radish leaf	0.800	10.93	3.430	0.046	0.2967
Onion	34.5289	0.178	1.29	0.026	0.0050
Mushroom	29.3824	1.60	13.63	0.030	0.0500

[a] Griess reagent; absorbance at 526 nm.
[b] 6.068 g $NaNO_3$ in 1 L (or 1.517 g in 250 mL), diluted × 100.

20 min in darkness (absorption maximum at 526 nm).

(2) 1 mL *N*-1-naphthylethylenediamine dihydrochloride solution and 1 mL sulfanilamide solution (7, 8) added in order. A deep red stable color is obtained immediately after mixing (absorption maximum at 539 nm).

The absorbance of the colored solutions was measured through 1 cm path length Hellma quartz cells with a Zeiss spectrophotometer PM 2A against distilled water. Results were recorded as μg total nitrites. Blank readings were obtained from 5 to 25 mL diluted buffer solutions passed through the Cd column or not, adjusted to 50 mL, and diazotized. The presence of original nitrite is detected with the same procedure of extracting, deproteinizing, buffering, and colorimetric reaction, but deleting the Cd column treatment.

Nitrate concentration was calculated by subtracting original nitrite readings from total nitrites, as demonstrated above.

Aspects Concerning Calculation of Nitrate (Nitrite) Content

The relationship between the measured absorbances versus the respective primary aliquots of the food extract and/or the $NaNO_3$ solution containing 10 μg NO_3-N/mL is linear. The straight lines obtained from the calibration curve and the unknown solution may be expressed as

$$y_c = ax_c + z_c \text{ and}$$
$$y_s = bx_s + z_s$$

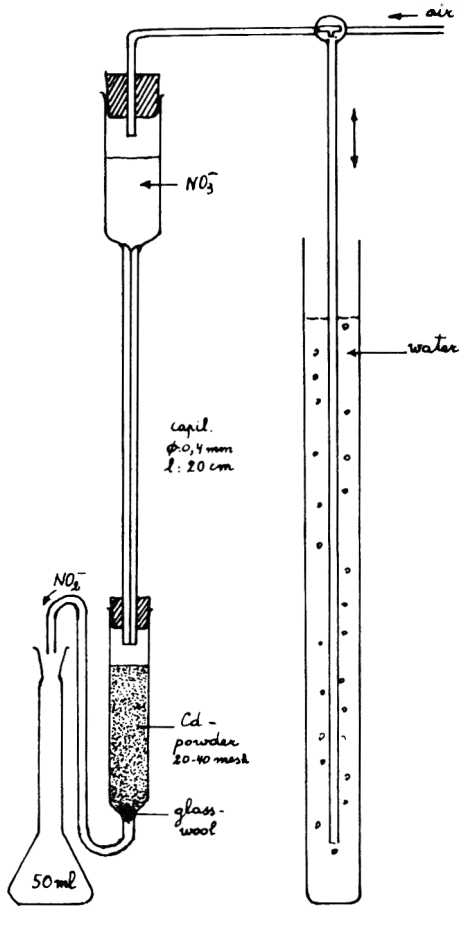

Figure 1. Cadmium reduction column connected to hydro-pneumatic system for constant pressure.

where y = measured absorbance; x = pipetted aliquot of food extract or $NaNO_3$ solution (expressed in mL); z = theoretical value of absorbance at zero sample volume (infinite dilution of extract); a, b = curvature (slope) calculated by linear regression from 6 measured absorbance values obtained from one food extract; c, s = calibration solution and sampled food extract. Normally z_c, z_s, Cd blank, and water blank values are expected to be equal. Table 1 demonstrates that these values can differ from one food extract to another, which is probably the main reason why chemically determined NO_3^- contents by standard methods differ within each other. To avoid this sample dependency, the following considerations can be taken into account:

In standard methods, the absorbance value (y_s − water blank) instead of the real ($y_s - z_s$) value is compared on a calibration curve to find the corresponding NO_3^- concentration. We may assume that equal NO_3^- quantities are present in both the food extract aliquots and the $NaNO_3$ solution aliquots when absorbance readings are equal after the prescribed treatment, or when ($y_c - z_c$) = ($y_s - z_s$), or when $a.x_c = b.x_s$. For $x_s = 1$ mL, we find $x_c = (b/a) \times 1$ (mL). This fraction (b/a) of the calculated curvature expresses also that 1 mL of the food extract under investigation contains as much nitrates as x_c mL of the standard $NaNO_3$ solution containing 10 μg N-NO_3/mL.

Also the N-NO_3 concentration of the food extract can be calculated by multiplying x_c by 10; this is (b/a) × 10.

By the fact that b and a represent curvatures, the calculated concentration $b/a \times 10$ is independent of the suspect zero and/or blank values.

This also means that the calculated concentration depends only on the curvatures and will not change if an equal amount of nitrates (1 mL of 10 μg/mL solution, for example) is added to each food extract before treatment through the Cd column.

Figure 2 demonstrates that the absorbance values plotted as a function of the pipetted aliquots with increasing volumes under a constant NO_3^- addition or not, result in parallel lines. The intercept $\Delta(i_2 - i_1)$ of the 2 lines with the calibration curve depends on the added amount of nitrate. If we should on the other hand investigate a series of solutions with increasing NO_3^- concentrations, obtained by nitrate addition from one food extract, we will find a fan of lines with a constant extrapolated absorbance value for zero volume but with an increasing curvature in respect to the increasing concentration.

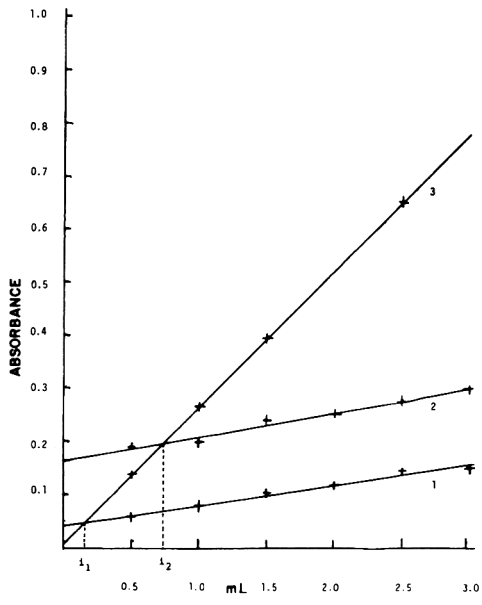

Figure 2. Absorbance as a function of sample volume, Griess reagents and red cabbage extract: 1, original extract; 2, original extract after addition of 5 μg NO_3-N to each pipetted aliquot; 3, calibration curve. (Drafted on Hewlett Packard Instruments calculator.)

To demonstrate this, we made a series of solutions with increasing NO_3^- concentration from a red cabbage extract. Each solution is analyzed for NO_3^- as described before. The results for the Griess colorimetric method are plotted in Figure 3.

The results of the nitrate determinations on a series of original extracts of radish leaves and with nitrate addition as described before, using both coloring methods, are summarized in Table 2.

Similar series of experiments were repeated for other vegetable extracts such as red cabbage, spinach, and salads. Preliminary studies showed that the ratio of the slopes of the calibration lines for both colorimetric methods were constant during the experiments, although the ratio of the corresponding slopes for the extracts may be different depending on the vegetable.

The origin of these differences will be reported in detail elsewhere. A summary of ratios and C_A/C_B values for both radish leaves and red-cabbage extracts as a function of the NO_3^- concentration is shown to demonstrate the limits of applicability of both colorimetric methods (Table 3).

From these results it can be deduced that low

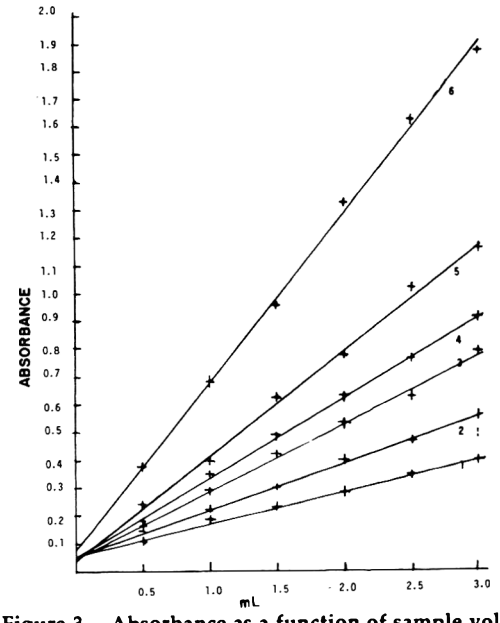

Figure 3. Absorbance as a function of sample volume, Griess reagents and radish leaf extract (4 g/250 mL): 1, original extract diluted (10 → 25 mL); 2, original extract diluted (10 → 25) + supplemental nitrate to 2 μg/mL; 3, original extract diluted (10 → 25) + supplemental nitrate to 5 μg/mL; 4, calibration curve; 5, original extract diluted (10 → 25) + supplemental nitrate to 10 μg/mL; 6, original extract diluted (10 → 25) + supplemental nitrate to 17.5 μg/mL. (Drafted on Hewlett-Packard Instruments calculator.)

concentration levels (<1 μg/mL) can be determined more accurately by using the second colorimetric method; for higher concentrations (>20 μg/mL), however, the Griess colorimetric method (method A) seems to be more accurate and reproducible.

From our experiments we deduce that even extracts with low nitrate levels can be determined by using the increasing sampling method with addition of a constant amount of nitrates. Only the slope of the final line (parallel with that of the original extract) determines the concentration of the original solution (cf. Figure 2 and Table 2).

Conclusions

Our results demonstrate the applicability of the proposed method for nitrate determinations. Advantages of the procedure include: (a) an extended apparatus with controlled parameters; (b) an improved sampling of plant extracts—instead of repeating the work on a constant aliquot and referring the obtained absorbance to a calibration curve for the determination of the concentration, we sample the vegetable extract with increasing volumes and calculate the final concentration from the slopes of the obtained absorbance/volume curves of both solutions: (*1*) solution with known NO_3^- concentration, (*2*) extract of the investigated vegetable. This way makes the NO_3^- (or NO_2^-) determination independent of blank values, which are a source of errors especially at low concentrations. Furthermore, low nitrate levels can be determined accurately by using the sulfanilamide-*N*,1-naphthylethylenediamine dihydrochloride dia-

Table 2. Nitrate determinations in radish leaf extracts

Object	Calibr. curve	Original soln	Original + 10 μg	II[a]	II[a] + 0.5 μg/mL	II[a] + 2 μg/mL	II[a] + 5 μg/mL	II[a] + 10 μg/mL	II[a] + 17.5 μg/mL
				Griess Reagent					
z	0.0512 7	0.0452	0.3639	0.0591	0.0818	0.05360	0.04420	0.0404 7	0.0730
b_A	0.2714 0	0.2967	0.2720	0.1146	0.1295 4	0.1685 1	0.2438 9	0.3772 6	0.6080
Correl.	0.9994 1	0.9999 0	0.9996 0	0.9981 0	0.9875 9	0.9993 0	0.9980 4	0.9970 8	0.9905 7
Concn A, μg/mL	10.0	10.93	10.02 + . . .	4.22	4.727	6.209	8.986	13.90	22.40
				N-1-Naphthylethylenediamine Dihydrochloride					
z	0.1113 3	0.0509 3	0.7602 0	0.0877 3	0.0510 7	0.0872 7	0.0525 3	0.0777 0	0.2305 0
b_B	0.5482 9	0.5922 3	0.5032 0	0.2314 9	0.2549 1	0.3422 3	0.4941 7	0.7542 0	0.9790
Correl.	0.9982 1	0.9990 3	0.9992 8	0.9974 0	0.9916 7	0.9964 3	0.9987 1	0.9976 7	—
Concn B, μg/mL	10	10.80	9.18 + . . .	4.22	4.65	6.24	9.01	13.75	17.8
b_A/b_B	0.4949 9	0.5009 9	0.5405	0.4950 5	0.5081 8	0.4923 9	0.4935 3	0.5002 1	0.6210
Concn A/ concn B	1	1.012	—	1.000	1.016	0.9950	0.9972	1.0105	1.254

[a] II: Original extract diluted 10 mL → 25 mL.

Table 3. Nitrate determination in extracts of radish leaves and red cabbage

Term	Concentration, μg/mL							
	Radish Leaves							
	10[a]	4.22	4.69	6.22	9.0	10.9	13.8	22.4
$\frac{b_A}{b_B}$	0.49499	0.4950	0.5082	0.4924	0.4935	0.5010	0.5002	0.6210
$\frac{C_A}{C_B}$	1.000	1.000	1.016	0.9950	0.9972	1.012	1.010	1.25
	Red Cabbage							
	10[a]	1.80	1.50	1.97	2.45	3.68	4.77	6.88	12.6
$\frac{b_A}{b_B}$	0.527 36	0.4532	0.5308	0.5199	0.5346	0.5208	0.5157	0.5288	0.5179
$\frac{C_A}{C_B}$	1.000	0.8597	1.009	0.9864	1.014	0.9883	0.9780	1.002	0.9819

[a] Solution of $NaNO_3$ containing 10 μg NO_3^--N/mL.

zotization mixture or by adding a constant amount of nitrates to the series of sampled volumes to reach a suitable nitrate range.

Because blank values for NO_3^- standard solutions and NO_3^--containing vegetable extracts can differ, it can be deduced that nitrate concentration determined by an automatically set blank can not be accurate.

Finally, our results show that the method is suitable for automated NO_3^- determination, in which absorbance readings are transmitted to a computer which calculates the concentration from slopes determined by linear regression.

REFERENCES

(1) Gruener, N., & Shuval, H. (1977) in *Developments in Water Quality Research*, Ann Arbor Science Publ. Inc., Ann Arbor, MI, pp. 89–106
(2) Herrmann, K. (1972) *Ernaehr. Umsch.* **11**, 398–402
(3) Schuphan, W., & Schlottmann, H. (1965) *Z. Lebensm. Unters. Forsch.* **128**, 71–75
(4) Lox, F. (1980) *Forum Ware* 8(1), 35–37
(5) Volff, R., Noyelle, G., & Gautrat, C. (1974) *Ann. Falsif. Expert. Chim.* **67**, 599–608
(6) Kamm, L., McKeown, G. G., & Smith, D. M. (1965), *J. Assoc. Off. Agric. Chem.* **48**, 892–897
(7) Okabe, A. (1975) *Hikone Ronso* **174**, 82–92
(8) Hamilton, J. E. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 284–288

Determination of Polysorbate 60 in Salad Dressings by Colorimetric and Thin Layer Chromatographic Techniques

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Methods are described for the quantitation and verification of polysorbate 60 (PS 60) in salad dressing. The sample is partitioned between methylene chloride and water. The methylene chloride extract is further treated by silica gel column chromatography. The isolated PS 60 is complexed with ammonium cobaltothiocyanate and determined spectrophotometrically at 620 nm. Additional evidence indicating the presence of PS 60 is obtained from thin layer chromatographic analysis using the modified Dragendorff reagent for visualization of spots.

Polysorbate 60 (PS 60), which is a complex mixture of polyoxyethylated fatty acid esters of sorbitol and its anhydrides, is widely used as a food additive and processing aid. The Code of Federal Regulations (CFR) limits the use of PS 60 in foods to concentration levels of 1% or lower, depending on the food product (1). The limit for PS 60 in salad dressings is 0.3% (3 mg PS 60/g sample).

A number of analytical methods for the determination of PS 60 have been described in the literature. Murphy and Scott (2) used a Soxhlet apparatus to extract PS 60 from baked goods and fat followed by detection with thin layer chromatography (TLC). Smullin et al. (3) analyzed several food matrices by saponifying the polysorbate and precipitating the resulting polyol with barium phosphomolybdate. Lindner (4) quantitated the fatty acid ester portion of the polysorbate molecule via gas chromatography. Boyer et al. (5) quantitated polyoxyethylated detergents in environmental samples, using the ammonium cobaltothiocyanate complexation procedure. These methods, if applied to complex food matrices, would require time-consuming sample preparation to eliminate interfering compounds. We believed that a more selective method requiring minimal sample preparation was needed.

In this paper we describe a method for the determination of PS 60 in salad dressings with a combination of silica gel column and colorimetric techniques. Although PS 60 is authorized for use in a wide variety of foods, we limited our work to salad dressings because of the extensive use of this additive in salad dressings of all types. Not only is this matrix sufficiently complex to provide a challenge for the method, but it is also free from the complications of thermal decomposition, which can occur in heated products such as baked goods. A TLC procedure, using the modified Dragendorff reagent as discussed by Murphy and Scott (2), is also described and is used to substantiate the results of the colorimetry.

The colorimetric technique, which is based on the reactions of specific functional groups, the poly(oxy-1,2-ethanediyl) oligomers, was selected because PS 60 is a complex mixture consisting of many different compounds, all of which are completely or partially composed of polyoxyethylene chains. These substances comprising PS 60 fall into 2 main categories: Tweens and carbowaxes. The Tweens consist of the sorbitan molecule with one or more fatty acid ester linkages as well as polyoxyethylene chains, with a total of 20 ethylene oxide equivalents per molecule. The carbowaxes include polyoxyethylene oligomers as well as sorbitan-derived substances which bear one or more polyoxyethylene chains (but no fatty acid esters). These polyoxyethylene chains are foreign to food but common to all the compounds found in PS 60.

The sample is partitioned between methylene chloride and water and the phases are separated. The methylene chloride fraction is concentrated and transferred to a silica gel column for purification. The silica gel column is washed with acetonitrile to remove interfering sample components. The PS 60 is eluted with a mixture of methylene chloride, methanol, and acetone. The eluate is evaporated and the residue is diluted to volume with methylene chloride. The sample is then reacted with ammonium cobaltothiocyanate reagent and the color intensity is measured at 620 nm. The results are verified by the TLC detection procedure with modified Dragendorff reagent.

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METHOD

Apparatus

(a) *Spectrophotometer.*—Visible and ultraviolet (UV) range. Cary Model 118 (Varian Associates, Palo Alto, CA 94303), or equivalent.

(b) *Glass column.*—25 cm Kontes Chromaflex No. K420280, 17 mm od, 14.5 mm id (Kontes Co., Vineland, NJ 08360).

(c) *TLC developer.*—Camag Vario KS (Camag Inc., 16229 W Ryerson Rd, New Berlin, WI 53151), equipped with 18 × 2.5 cm solvent chamber and with 18 × 18 cm solvent tray (for saturation of atmosphere near plate).

(d) *Solvent wicks for TLC developer.*—Camag Inc.

(e) *Precoated TLC plates.*—Silica gel 60, 20 × 20 cm, 0.25 mm layer thickness (available from Scientific Products Div., American Hospital Supply Corp., McGaw, IL 60085).

(f) *TLC sprayer.*—Supelco Cat. No. 5-8005 (Supelco, Inc., Bellefonte, PA 16823), or equivalent.

(g) *Shortwave ultraviolet lamp.*—Chromato-Vue cabinet (Ultra-Violet Products, Inc., San Gabriel, CA 91778), or equivalent.

(h) *Rotary evaporator.*—Equipped with ₹ 24/40 ground glass joints (Buchler Instruments Inc., Fort Lee, NJ 07024).

(i) *Temperature bath.*—Buchler Instruments Inc.

Reagents

(a) *Solvents.*—Methanol, methylene chloride, acetonitrile, and acetone. Distilled-in-glass quality (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

(b) *Ammonium cobaltothiocyanate reagent.*—Dissolve 62 g ammonium thiocyanate, 28 g cobalt nitrate, and 50 g sodium chloride (all reagents certified ACS, Fisher Scientific Co., Pittsburgh, PA 15219, or equivalent) in 250 mL water. Dilute to 500 mL with water. Extract impurities with 100 mL methylene chloride.

(c) *Polysorbate 60 (PS 60).*—Samples from the following suppliers gave equivalent colorimetric responses and identical TLC patterns: Emery Industries, Inc., PO Box 628, Mauldin, SC 29662; Glyco Chemicals, Inc., Williamsport, PA 17701; Hodag Chemical Co., 7247 N Central Park Ave, Skokie, IL 60077; ICI Americas, Inc., Atlas Chemical Division, Wilmington, DE 19810; PVO International, Inc., 416 Division St, Boonton, NJ 07005.

(d) *High vacuum grease.*—Dow Corning Corp., Midland, MI 48640.

(e) *Silica gel.*—7% deactivated (Fisher Scientific Co.). Heat silica gel at 100°C in vacuum oven for ≥4 h. To 100 g silica gel add 7 mL water; mix thoroughly. Store in airtight jar.

(f) *Centrifuge.*—Sorvall RC-3 (DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Newtown, CT 06470), or equivalent, equipped to hold 50 mL test tubes.

(g) *Vacuum oven.*—National Appliance Co., Portland, OR 97204, or equivalent.

(h) *Modified Dragendorff reagent.*—Dissolve 1.7 g bismuth subnitrate (Scientific Products Div.) in 20 mL acetic acid; some heating may be necessary to obtain clear solution. Add 80 mL water and solution of 40 g potassium iodide in 100 mL water. Add 200 mL acetic acid, label as Solution A, and store solution in dark. Prepare Solution B by dissolving 20 g barium chloride in 75 mL water and diluting to 100 mL with water. These solutions are stable for 2 weeks. Prepare spray immediately before use by mixing 2 parts Solution A with 1 part Solution B (6).

(i) *Sodium nitrite solution.*—Dissolve 5 g sodium nitrite in 95 mL water (7).

Sample Extraction and Liquid-Liquid Partition

Weigh 5 g sodium chloride in 250 mL separatory funnel. Add 5 g salad dressing, 125 mL methylene chloride, and 25 mL water. Shake vigorously 200 times. (If he desires, the analyst may note time required to shake vigorously 200 times and perform subsequent extractions on basis of that time.) Let layers separate and drain methylene chloride layer into 200 mL volumetric flask. Re-extract aqueous layer with 75 mL methylene chloride. Shake vigorously 200 times. Combine methylene chloride extracts in 200 mL volumetric flask. Dilute to 200 mL with methylene chloride. Quantitatively transfer 150 mL of sample extract to 250 mL round-bottom flask. Set remainder of sample aside for TLC analysis. Use rotary evaporator with water aspirator vacuum to reduce solution volume to 5–15 mL.

Silica Gel Chromatography

To glass column add 7 g silica gel. Prewash column with 25 mL acetonitrile. Drain acetonitrile from column until meniscus is 1 mm above top of silica gel packing. Transfer sample to column, using four 25 mL acetonitrile rinses. After each addition, drain column, until meniscus nearly touches top of column packing. Elute PS 60 from column with 150 mL methylene chloride–methanol–acetone (55 + 30 + 15) and

Table 1. Spiking studies of polysorbate 60 in salad dressings[a]

Level, % (w/w)	Av. rec., %	CV, %
0.1	82	3.7
0.3	85	2.4
1.0	86	1.3

[a] 9 determinations at each level.

collect in 250 mL round-bottom flask. Remove all solvent on rotary evaporator and immediately dissolve residue in ca 20 mL methylene chloride. Quantitatively transfer to 50 mL volumetric flask and dilute to volume.

Standard Curve

Pipet 1, 2, 4, 8, 15, and 30 mL aliquots of PS 60 standard stock solution (3 mg PS 60/mL methylene chloride) into separate 100 mL volumetric flasks and dilute to volume with methylene chloride to obtain standard concentrations of 0.03, 0.06, 0.12, 0.24, 0.45, and 0.90 mg/mL, respectively. Pipet 20 mL of each standard solution into separate 60 mL separatory funnels. Quantitatively add 20 mL ammonium cobaltothiocyanate reagent to each funnel and shake vigorously 100 times. (If he desires, the analyst may note time required to shake vigorously 100 times and perform subsequent analyses on basis of that time.) Centrifuge at 1500 rpm for 20 min. Measure absorbance at 620 nm. (Color begins to fade significantly after 1/2 h.) Plot standard curve of absorbance vs concentration.

Determination

Pipet 20 mL aliquot of purified sample into 60 mL separatory funnel. Carry out colorimetric procedure as described for standards and measure absorbance at 620 nm. Read concentration of PS 60 from standard curve. Calculate amount PS 60 in sample as follows:

$$C = C' \times (50/150) \times (200/W)$$

where C is the concentration of PS 60 in the sample (mg/g), C' is the concentration of the sample from the standard curve (mg/mL), and W is the weight of the sample (g).

TLC Analysis

Score silica gel TLC plate into eight 2 × 20 cm channels. About 1 in. from base of a channel, spot enough sample to deposit 6 μg polysorbate. Up to 400 μL of sample extract may be spotted. On one side of sample, spot 5.0 μg polysorbate standard; on the other side, spot 8.0 μg polysorbate standard. Fill solvent chamber and solvent tray of TLC developer with methylene chloride. Position plate in TLC developer. (A TLC tank instead of the TLC developer was used for this step.) Develop to within 2 cm of end of TLC plate with methylene chloride to remove oil from spots. Place plate in hood for 1 h to permit evaporation of methylene chloride. View plate under UV light. If the region between the origin and 8 cm above the origin is not free of fluorescence-quenching material, redevelop in methylene chloride to within 2 cm of top of TLC plate. Repeat drying process. Fill solvent chamber and solvent tray of TLC developer with methylene chloride–methanol–acetone–water (55 + 20 + 15 + 4). Position plate in TLC developer and develop 8–10 cm. After development, spray with modified Dragendorff reagent (6). Four spots will be obtained. Dragendorff spray turns the Tweens (less polar spots) orange and the carbowaxes (more polar spots) pink. Spray with sodium nitrite solution after the Dragendorff spray to enhance polyoxyethylene detection (7).

Results and Discussion

We spiked a PS 60-free commercial salad dressing at levels of 0.1, 0.3, and 1.0%; recoveries averaged 82, 85, and 86%, respectively, as determined by colorimetric analysis. These results are shown in Table 1.

We also analyzed 6 stable emulsion dressings, each of which included PS 60 as an ingredient. These results, which are given in Table 2, were substantiated by TLC. Four spots for each sample were obtained with R_f values 0.5, 0.6, 0.7, and 0.8; some streaking, which occurred in between the spots, was due to the complexity of the PS 60 mixture. Because R_f values are somewhat variable, reference standards were included on each plate. We used Dragendorff spray to visualize

Table 2. Polysorbate 60 in stable emulsion dressings[a]

Dressing	Polysorbate 60, mg/g[b,c]
Creamy italian—brand A	2.6
Creamy italian—brand B	1.7
Russian	0.9
Thousand island	1.0
Low calorie french	2.3
Creamy cucumber	1.7

[a] Values semiquantitatively confirmed by TLC.
[b] Results were corrected on the basis of the 84.4% mean recovery for the spiking studies.
[c] Duplicate determinations.

the spots. From our observations, the TLC procedure was accurate to within 20% of the values determined in the colorimetric procedure. Therefore, we bracketed each sample spot with standards which were ±20% of the analytical value.

To determine which spots in the PS 60 were Tweens and which were carbowaxes, we used the Wiebull procedure as described by Coupkova et al. (8). The sample is partitioned between aqueous sodium chloride and ethyl acetate. The Tweens are retained in the ethyl acetate layer and the carbowaxes enter the aqueous layer. We found the carbowaxes to be the 2 most polar spots (lower R_f values) and the Tweens, the 2 least polar spots (higher R_f values).

We used the TLC procedure to ensure that all of the PS 60 was eluted from the silica gel column with a 150 mL volume of mobile solvent. We passed six 25 mL aliquots of methylene chloride–methanol–acetone (55 + 30 + 15) through the column and spotted an aliquot of each fraction on a silica gel TLC plate. No polysorbate was detected in the last fraction.

The techniques reported above permit the analysis of PS 60 in a variety of salad dressings. The silica gel column/complexiметric method provides rapid quantitation of the ethoxylates in the sample. The specificity of the method is due in part to the fact that the column can be washed with a polar solvent such as acetonitrile to remove interfering sample components before the PS 60 is eluted with the methylene chloride–methanol–acetone solvent mixture. Further evidence of the identity of PS 60 can be obtained with the TLC system described.

REFERENCES

(1) *Code of Federal Regulations* (1981) Title 21, U.S. Government Printing Office, Washington, DC, sec. 172.836
(2) Murphy, J. M., & Scott, C. C. (1969) *Analyst* **94,** 481–483
(3) Smullin, C. F., Wetterau, F. P., & Olsanski, V. L. (1971) *J. Am. Oil Chem. Soc.* **48,** 18–20
(4) Lindner, W. (1974) *J. Chromatogr.* **101,** 57–62
(5) Boyer, S.L., et al. (1977) *Environ. Sci. Technol.* **11,** 1167–1171
(6) Stahl, W. (Ed.) (1969) *Thin-Layer Chromatography, a Laboratory Handbook*, Springer-Verlag, Inc., New York, NY, p. 873
(7) Hodda, A.E. (1976) *J. Chromatogr.* **124,** 424–425
(8) Coupkova, M., Janes, K., Sanitrak, J., & Coupek, J. (1978) *J. Chromatogr.* **160,** 73–80

OILS AND FATS

Gas Chromatographic-Mass Spectrometric Analysis, Identification, and Detection of Adulteration of Natural and Concentrated Lemon Oils

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Lemon oils were characterized by quantitative glass capillary gas chromatography. Components present to the extent of more than 0.05% were measured and identified by mass spectrometry. In expressed lemon oils, monoterpenes and geranial were the only components present at concentrations of 1.0% or more. In concentrated lemon oils, β-caryophyllene, linalool, neral, α-terpineol, neryl acetate, and geranyl acetate were present in some samples in amounts of 1% or more. Adulteration was detected by component concentrations outside characteristic ranges for oils judged to be authentic.

For the assessment of the proper rate of duty on importations of essential oils, it is necessary to correctly identify these commodities and detect any adulteration. Work performed with several different perfumery materials including lavender and lavandin oils (1, 2) and products from the bitter orange tree (3) has shown that this can be accomplished by quantitative determination of components present to the extent of about 0.5% or more in these complex mixtures. Because the concentrations of the compounds present in these natural products vary to some extent, it is useful to analyze numerous samples and determine concentration ranges for the major components of the oils. Adulterated oils have been recognized frequently by concentrations of important components that fall outside of the established ranges. Glass capillary column gas chromatography is a satisfactory technique for the analysis of essential oils. The use of packed columns, on the other hand, has provided insufficient separation of components for adequate quantitative analysis of these commodities.

Natural and concentrated lemon oils are commonly imported into this country. Many analytical studies of lemon oils have been reported in the literature, primarily providing concentration data for monoterpenes, neral, and geranial, the predominant components of lemon oil. Often the analysis was performed on preseparated fractions of the oils. Relatively few papers (4–9) give a more detailed quantitative analysis performed on the complete oil. For best accuracy, analysis of the complete oil is preferred to analysis of fractions obtained by preliminary separations (10). Although glass capillary chromatography is the method of choice for the determination of citrus oil components (11), only one of the quantitative studies of lemon oils was reported (9) to have been performed with a capillary column. The analysis of concentrated lemon oils has not been reported and the studies of natural oils at times involved only one sample (6–8) or achieved poor separation of even the most important components (4). Consequently, there is actually insufficient analytical information available for judging the authenticity of lemon oils.

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METHOD

Apparatus and Reagents

(a) *GLC column.*—33 m × 0.5 mm id SCOT glass capillary column containing Carbowax 20M stationary phase (Scientific Glass Engineering Inc., 2800 Longhorn Blvd, Austin TX 78759).

(b) *Gas-liquid chromatograph.*—Perkin-Elmer Model 3920, equipped with flame ionization detector and splitting injector providing a split ratio of 30:1. Operating conditions: initial temperature of 60°C held 4 min, programmed at 2°/min to 200°C, and held 32 min; injector 225°C; detector 250°C; helium carrier gas 19 cm/s; hydrogen 40 mL/min; air 550 mL/min.

(c) *Mass spectrometer*—Hitachi-Perkin-Elmer Model RMU-6L, single focus, low resolution coupled to chromatograph with jet separator. Operating conditions: ionization voltage 70 eV; source temperature 200°C; interface 250°C; target current 50 μA; accelerating voltage 3200 V.

(d) *Data system.*—Hewlett-Packard Model 3352 B.

(e) *pH meter*—Leeds & Northrup Model 7415 with glass and saturated calomel electrodes.

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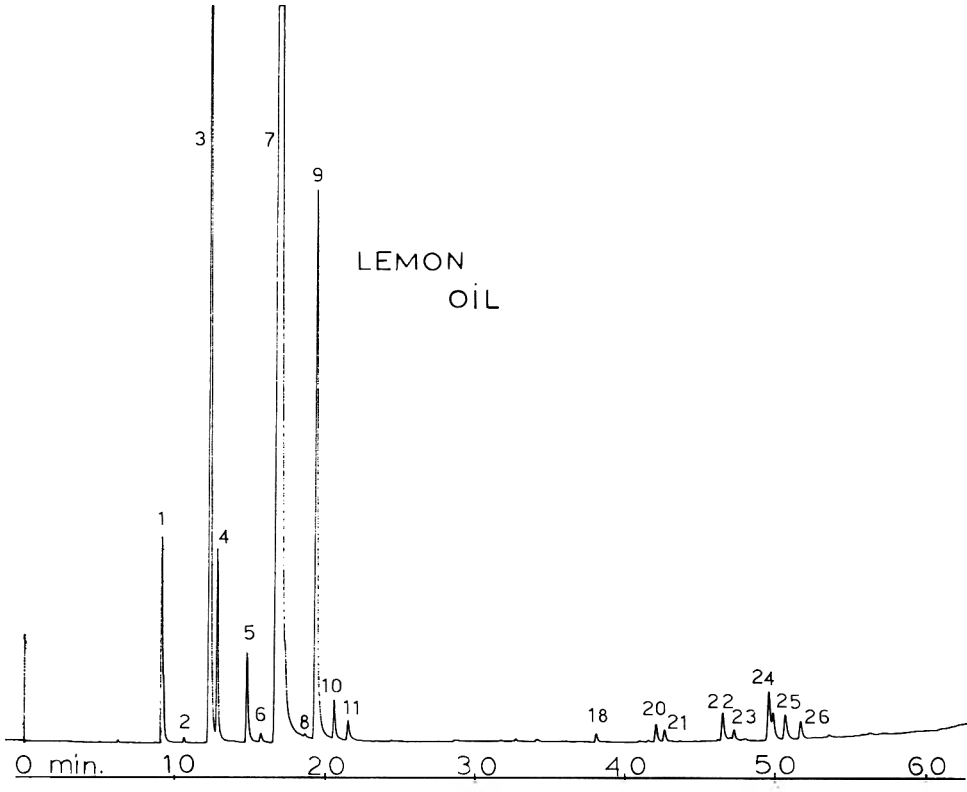

Figure 1. Chromatogram of expressed lemon oil. See Table 1 for identification of peak numbers.

(f) *Essential oils.*—Imported samples submitted for Customs examination and reference samples from Berje Chemical Products Inc., New York, NY, and Dragoco, Inc., Totowa, NJ 07511.

(g) *Essential oil components.*—Camphene, myrcene, neral, geraniol, citral (Aldrich Chemical Co., Inc., Milwaukee, WI 53233); limonene, γ-terpinene, linalool, linalyl acetate, linalool oxide (IFF Inc., Hazlet, NJ 07730); terpineol-4 (Applied Science Laboratories, Inc., State College, PA 16801); α-terpineol (Eastman Kodak Co., Rochester, NY 14650); α-terpinene (PCR Research Chemicals, Inc., Gainesville, FL 32602); *p*-cymene (Fritzsche Dodge & Olcott Inc., New York, NY 10011); β-caryophyllene (Roure Bertrand DuPont, Teaneck, NJ 07666); β-pinene (Procter & Gamble, Cranford, NJ 07016); terpinolene (Colgate Palmolive Co., Piscataway, NJ 08854).

(h) *Hydroxylamine hydrochloride reagent.*—0.5N. Dissolve 17.4 g $NH_2OH{\cdot}HCl$ (AR grade) in isopropyl alcohol and dilute to 500 mL.

(i) *Sodium hydroxide reagent*—0.5N. Dissolve 20.0 g NaOH (AR grade) in water, dilute to 1 L, and standardize.

Procedure

(a) *Gas chromatography–mass spectrometry.*—Inject 0.2 μL sample or reference compounds directly into gas chromatograph. Measure peak areas of the major components of oils for quantitation by attenuating the chromatogram and determine retention times relative to linalool = 1 for identification. For confirmation, measure mass spectra of separated components and compare with spectra or reference materials or published data (12–14).

(b) *Total aldehyde determination.*—Place 50 μL $NH_2OH{\cdot}HCl$ reagent in 125 mL Erlenmeyer flask and add enough sample to require about 20 mL titrant (10 mL samples of concentrated lemon oils were used). Let stand at room temperature 30 min. Rinse solution with minimum amount of isopropyl alcohol into 150 mL beaker and titrate potentiometrically with standard 0.5N NaOH solution.

Results and Discussion

Of 41 samples analyzed, 31 are considered to be natural lemon oils. A typical chromatogram

obtained with a sample of one of these oils is shown in Figure 1. Of the remaining 10 samples, 6 are considered to be concentrated and 4 are adulterated oils. Concentration data based on area percent for the chromatographically separated components and identification of these based on relative retention times (RRT) and mass spectroscopic analysis are listed in Table 1.

In the natural oils, the same 17 peaks each representing 0.06% or more of the total area were found in almost all of the samples, and concentration data for these compounds are given in Table 1. These components accounted for 97.4–100% and 99.4% on the average of the composition of the volatile portion of the oils. The nonvolatile components of lemon oil account for up to 2% of the total oil (10). One other peak, geranyl acetate (RRT = 1.43), was found in about one half of the samples, and 2 additional small peaks with RRT of 0.46 and 0.53 were found in about one third of the samples. These latter 2 peaks were very close to other peaks with RRT 0.47 and 0.52, respectively, and may not have been separated from these larger peaks in most of the analyses. No other peak was found in more than 6 samples.

In these 31 chromatograms, only 41 additional peaks with RRT of 1.5 or less were recorded. Twenty-nine of these peaks corresponded to 0.1% and only 2 were as large as 0.4%. There were also 24 small peaks with RRT greater than 1.5.

The concentrations of the components found in lemon oil fell into narrow ranges. Only one component concentration was significantly different from those observed in the other oils, one sample contained 0.9% linalool compared with no more than 0.3% in the other samples. Nine terpenes and geranial were the only components present in these samples to the extent of 1% or more and also averaged at least 1.0%. Neral was the only other compound present in concentrations greater than 0.5% in any sample. Limonene is the main component in lemon oil and its concentration in these samples averaged 67%. Concentrations of β-pinene and γ-terpinene averaged about 10% each. The results obtained in these experiments agree well with those found in the literature (4–9), except that some data (5.0% α-pinene and 2.9% γ-terpinene) by Fincke and Maurer (7) differ substantially from those found in this work and by others.

The main sources of these 31 samples were Argentina, 11 samples, and Italy, 6 samples. No significant difference in the concentration of any component was observed that could be attributed to country of origin. It has been reported (9) that lemon oils from Italy contain a little more citral (geranial plus neral) than oils from Argentina, about 3–4% and 2–3%, respectively. Somewhat higher citral values were obtained in this work also for Italian oils, 2.2-3.1% compared with 1.4–2.6% for oils from Argentina, but they were not statistically different when examined by the Student t-test (15).

The citral content is an important factor which influences the quality of lemon oils (10, 16). Concentrated lemon oils contain larger amounts of aldehydes and other oxygenated compounds and smaller quantities of terpenes than do ordinary expressed oils. Flash distillation is one process used to remove the terpenes with lower boiling points and thereby increase the relative amounts of citral and other oxygenated compounds. A reference sample of such an oil was analyzed and a commercial sample labeled lemon oil was apparently treated the same way, since its composition was almost identical to that of the reference sample. Terpenes that elute before limonene are almost entirely absent from these 2 samples, whereas they comprise more than 17% on the average of ordinary lemon oils. The limonene content of these 2 samples is only about 70% of that found in natural lemon oils. Later eluting components on the other hand are present in much higher concentrations than in natural lemon oils, e.g., γ-terpinene, β-caryophyllene, neral, α-terpineol, geranial, neryl acetate, and geranyl acetate.

Four other samples were also concentrated lemon oils, but processed differently. These oils imported by one company over a period of several years are concentrated by solvent washing, in order to avoid exposure of the products to heat. The data obtained for these oils indicate that by this process the concentration of all terpenes is decreased (including γ-terpinene and β-carophyllene) approximately to the same extent, about 20%. The citral content is increased to about 15%. The chromatograms of these samples contained 3 other peaks, including up to 0.3% each of nerol and geraniol. Two of the samples contained up to 0.5% of these 2 alcohols and had 4 other common peaks equivalent to 0.1–0.5% and RRT of up to 1.5.

Samples 1–4 of Table 1 are considered to be adulterated. Sample 1 contains more than 30% citral, compared with about 3% in natural lemon oils and about 15% in concentrated oils. Linalool and α-terpineol concentrations of 1.7 and 1.0%, respectively, are also high. Terpene concentrations, on the other hand are within expectations, the low amount of limonene being due to

Table 1. Composition (%) of lemon oils

					Concentrated lemon oil								
		Natural lemon oil			Flash distn		Solv. washing		Adulterated oils				
Peak	RRT	Av.	Range	RSD (%)	Av.	Range	Av.	Range	1	2	3	4	Identity
1	0.25	2.2	1.8–3.6	18	0.0	0.0	2.1	2.0–2.1	1.6	0.7	4.4	1.5	α-pinene
2	0.27	0.1	0.0–0.1	—	0.0	0.0	0.1	0.0–0.1	—	0.2	—	0.2	camphene
3	0.32	10.5	6.1–15.0	20	0.1	0.1	9.2	9.1–9.2	8.5	2.9	1.2	6.7	β-pinene
4	0.33	3.1	1.5–4.6	27	0.0	0.0	2.3	1.3–3.2	1.9	0.5	0.6	1.9	sabinene
5	0.37	1.5	1.0–2.1	16	0.1	0.1	1.0	0.9–1.2	1.0	0.7	1.3	1.1	myrcene
6	0.38	0.2	0.0–0.5	—	0.1	0.0–0.1	0.2	0.2	0.2	2.4	0.8	1.5	α-terpinene
7	0.42	67.2	62.1–74.5	4.2	46.2	44.4–47.9	54.7	53.6–56.2	44.2	58.6	82.4	68.2	limonene
8	0.46	0.3	0.2–0.4	—	0.3	0.2–0.4	0.1	0.0–0.2	0.1	—	—	—	—
9	0.47	9.3	6.0–11.6	13	22.3	20.7–23.9	7.0	6.8–7.3	5.0	3.1	2.7	8.0	γ-terpinene
10	0.50	1.0	0.3–1.8	38	1.9	1.9	1.3	1.1–1.4	0.5	1.5	1.0	1.9	*p*-cymene
11	0.52	1.0	0.3–1.9	47	2.5	2.3–2.6	0.5	0.4–0.6	0.2	1.0	1.0	5.8	terpinolene
12	0.53	0.2	0.1–0.5	—	0.0	0.0	0.6	0.4–1.0	0.2	1.0	—	—	octanal?
13	0.58	0.0	0.0	—	0.0	0.0	0.2	0.2–0.3	—	—	—	—	—
14	0.70	0.0	0.0	—	0.3	0.3	0.5	0.3–0.6	0.2	0.2	—	—	nonanal?
15	0.81	0.0	0.0	—	0.0	0.0	0.1	0.0–0.1	0.1	0.1	—	—	*trans*-linalool oxide
16	0.86	0.0	0.0	—	0.2	0.1–0.2	0.4	0.4–0.5	0.2	0.4	—	—	*cis*-linalool oxide
17	0.91	0.0	0.0	—	0.2	0.1–0.2	0.1	0.0–0.2	—	—	—	—	decanal?
18	1.00	0.1	0.0–0.9	—	0.8	0.6–0.9	1.0	0.7–1.6	1.7	1.9	0.3	0.1	linalool
19	1.04	0.0	0.0	—	0.2	0.0–0.3	0.1	0.0–0.1	0.2	0.9	—	—	linalyl acetate
20	1.09	0.2	0.0–0.4	—	0.3	0.2–0.3	0.2	0.0–0.4	0.3	0.2	—	—	terpineol-4
21	1.12	0.2	0.0–0.5	—	3.9	3.2–4.5	0.3	0.3	0.1	0.2	0.1	0.3	β-caryophyllene
22	1.25	0.6	0.2–0.9	25	2.6	2.6	5.0	4.8–5.3	9.7	3.9	0.8	0.5	neral
23	1.27	0.2	0.0–0.4	—	1.4	0.7–2.8	1.2	0.8–1.5	1.0	0.8	0.2	—	α-terpineol
24	1.34	1.9	1.0–3.1	26	8.1	6.6–9.5	9.7	9.0–10.4	21.1	7.4	1.4	1.6	geranial
25	1.39	0.3	0.0–0.5	—	3.7	3.6–3.8	0.7	0.4–1.0	0.2	0.2	—	0.2	neryl acetate
26	1.43	0.1	0.0–0.3	—	2.7	2.3–3.1	1.2	0.0–1.9	0.2	0.3	—	—	geranyl acetate

Table 2. Comparison of aldehyde content determined by titrimetric and GC procedures

Method	Sample[a] 1	2	3	4
Av. aldehyde content, titr. method	10.0	17.6	18.7	13.7
Citral content, GC detn	7.6	12.6	14.0	10.5
Ratio, aldehyde content, titr. to citral content	1.32	1.40	1.34	1.30
"Total" aldehyde content, GC detn	8.0	14.4	15.3	11.7
Ratio, aldehyde content, titr. to "total" aldehyde	1.25	1.22	1.22	1.17

[a] Samples analyzed:
1 = a lemon oil concentrated by flash distillation
2–3 = lemon oils concentrated by solvent washing
4 = an adulterated oil.

dilution by citral. In Sample 2, the concentrations of β-pinene, γ-terpinene, and citral are far outside the ranges for normal lemon oils. The early eluting terpenes and the aldehydes are at concentrations consonant with those for concentrated oils, but the α-terpinene and linalool concentrations are too high and the γ-terpinene concentration is too low. The high linalyl acetate concentration is also an indication of adulteration, because this component is present only in traces in lemon oils (17). This sample also contained about a dozen unidentified late-eluting components with RRT greater than 2, equivalent to about 10% of the total area of the chromatogram. Samples 3 and 4 of Table 1 have normal concentrations of oxygenated compounds, but the distribution of hydrocarbons is unusual for lemon oils. The β-pinene and γ-terpinene concentrations, which average about 10% each and are at least 6% in normal lemon oils, are 1.2 and 2.7%, respectively, in Sample 3. The high α-pinene and α-terpinene concentrations and the normal quantities of oxygenated compounds rule out the possibility that this is a concentrated lemon oil. Sample 4 contained 3 times the amounts of α-terpinene and terpinolene found in any other sample.

As has been mentioned earlier, the aldehyde content is an important criterion of the quality of lemon oils and has long been cited for that reason. Although by nonchromatographic procedures total aldehyde content is measured, the results are usually expressed as amount of citral, which has been said to comprise 80–90% of the total aldehyde concentration of lemon oils (18).

Several of the samples were analyzed for total aldehyde. The most satisfactory procedure of a number that were tried involved reaction with hydroxylamine hydrochloride and potentiometric back-titration of the liberated HCl with standard NaOH (19). The data obtained are summarized in Table 2. The total aldehyde concentrations by titrimetry are the averages of 2–4 measurements, which exhibited an average relative standard deviation of 1.7%. These results were consistently one third higher than the corresponding neral plus geranial values obtained by gas chromatography.

Although several small peaks in Table 1 could not be identified by mass spectrometry, information in the literature (6, 9, 11, 20, 21) indicates that some of the minor components eluting between limonene and linalool may be aldehydes. A lemon oil was analyzed several times after being spiked with pairs of aldehydes. Results indicate that peaks with RRT 0.53, 0.70, and 0.91 represent octanal, nonanal, and decanal, respectively. If the concentrations of these components are combined with the neral plus geranial data and compared to the titrimetric results, the latter are uniformly a little less than one fourth higher than the GC data based on area percentages.

It is concluded that even without the use of response factors, the GC data for citral are generally adequate when used in conjunction with GC data for other components to determine whether a lemon oil is authentic or not. Natural lemon oils are easily identified by the characteristic concentrations of terpenes, neral, and geranial. Concentrated oils can be recognized by increased concentrations of several oxygenated compounds and changes in terpene concentrations, which depend on the processing of the oils.

REFERENCES

(1) Prager, M. J., & Miskiewicz, M. A. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1231–1238
(2) Prager, M. J., & Miskiewicz, M. A. (1981) *Perfum. Flavor.*, **6**(2), 53–58

(3) Prager, M. J., & Miskiewicz, M. A. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 131–138
(4) Bernhard, R. A. (1960) *J. Chromatogr.* **3,** 471–476
(5) Pennisi, L., & DiGiacomo, A. (1965) *Riv. Ital. Essenze Profumi, Piante Offic. Aromi-Saponi, Cosmet-Aerosol* **47,** 370–377
(6) Ziegler, E. (1971) *Flavour Ind.* **2,** 647–653
(7) Fincke, A., & Maurer, R. (1974) *Deut. Lebensm.-Rundsch* **70,** 100–104
(8) Lund, E. D., & Bryan, W. L. (1976) *J. Food Sci.* **41,** 1194–1197
(9) Dugo, G., Ragonese, C., & Licandro, G. (1977) *Essenze Deriv. Agrum.* **47,** 503–514
(10) Shaw, P. E. (1979) *J. Agric. Food Chem.* **27,** 246–257
(11) Wilson, C. W., III, & Shaw, P. E. (1980) *J. Agric. Food Chem.* **28,** 919–922
(12) Masada, Y. (1976) *Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry,* John Wiley & Sons, Inc., New York, NY
(13) *Eight Peak Index of Mass Spectra* (1974) 2nd Ed., Mass Spectrometry Data Centre, Aldermaston, Reading, UK
(14) Graselli, J. G. (Ed.) (1973) *Atlas of Spectral Data and Physical Constants for Organic Compounds,* CRC Press, Cleveland, OH
(15) Bauer, E. L. (1971) *A Statistical Manual for Chemists,* 2nd Ed., Academic Press, New York, NY, pp. 61–70
(16) Guenther, E. (1949) *The Essential Oils,* Vol. 3, D. Van Nostrand Co., Inc., New York, NY, pp. 96–105
(17) DiGiacomo, A. (1977) *Essenze Deriv. Agrum.* **47,** 463–472
(18) Retamar, J. A., et al. (1978) *Essenze Deriv. Agrum.* **48,** 135–149
(19) Siggia, S. (1963) *Quantitative Organic Analysis via Functional Groups,* 3rd Ed., John Wiley & Sons, Inc., New York, NY, pp. 73–79
(20) Bovalo, F., & DiGiacomo, A. (1977) *Essenze Deriv. Agrum.* **47,** 326–346
(21) DiGiacomo, A. (1978) *Labo-Pharma-Probl. Tech.* **26,** 508–514

FIBERS

Wax in Wool by Wide-Line Proton Magnetic Resonance: Rapid Non-Destructive Method

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A wide-line proton magnetic resonance technique (PMR), in which hydrogen response is used as the measure of wax in dried greasy wool, is a viable and preferred alternative to the commonly used Soxhlet procedure in which extracted weight is measured. The validity of the PMR method depends on wax hydrogen composition (inter- and intra-breed) being constant, and on background contribution from non-wax hydrogen (in suint, wool fiber, and vegetable matter) being small and constant. The experimentation reported in this paper shows that both requirements are met. The PMR method for wax is precise (better than 0.1% at mid-range); rapid (200 samples per 8 h day); sensitive (better than 15 mg); and yields a linear calibration graph over the entire range (0-100%) for a 5 g sample. A comparison of the methods shows that the linear regression line for wax by Soxhlet (y) against wax by PMR (x) is given by $y = 1.0257$ (SE 0.2277) + 0.9894 (SE 0.0142)x, with $r^2 = 0.99$, and $n = 20$.

Raw wool is a heterogeneous mixture of proteinaceous fiber coated with wax and suint and containing adsorbed and absorbed water. Vegetable matter and dust are present as contaminants. The wax content of wool is routinely measured because it is of interest in animal selection, in fleece rot investigations, and in processing and scouring. Soxhlet extraction with a hydrocarbon (lipid) solvent is the method commonly adopted. This procedure is time-consuming and of uncertain precision on a routine basis, requires large amounts of laboratory fume-hood space if batteries of extractors are used, and has the hazards associated with the distillation of volatile, flammable solvents.

To eliminate these problems, an instrumental method was considered. It was required of the alternative method that it be simple to perform and involve minimal sample preparation; that operator time per sample be considerably less than for the Soxhlet technique; and that it be at least as precise as the Soxhlet procedure and preferably of equal sensitivity.

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In our initial investigations of instrumental methods, it seemed that, in principle, an infrared method could, by judicious selection of characteristic peaks, determine quantitatively all the important components of wool (viz., wax, suint, water, wool fiber, and vegetable matter). In practice, however, we found no commercially available unit capable of satisfactorily performing these functions; because the problems in developing such a unit were assessed as formidable, we abandoned this approach.

Pulse nuclear magnetic resonance (NMR), which offered the possibility of simultaneous water and wax determinations, was also rejected after initial investigation because of the considerable developmental work required. The viable alternative seemed to be wide-line nuclear (proton) magnetic resonance (PMR), which has been used for such measurements as oil in seeds (1) and hydrogen in aviation fuel (2). This method, which would determine wax only, was investigated in detail.

Experimental Rationale

In the Soxhlet procedure (see below), wax is considered to be the weight of solvent-extractable material; in wide-line PMR, hydrogen response is proposed as the measure of wax. The Soxhlet procedure is known to be solvent-dependent and to be subject to carry-over of fines (dust, mold, bacteria, and some of the suint components).

Extensive studies have shown wool wax to be of complex and somewhat variable composition. The overall composition has, nevertheless, been established, and in a series of review articles Motiuk (3) has shown that lanosterol (11.8% H) and cholesterol (12.0% H) comprise about 75% of the wax alcohols.

Motiuk (4) has also shown that about 80% of wool-wax acids belong to 4 homologous series (normal, hydroxy, iso, and anteiso acids). Calculations from Motiuk's (4) typical acid for each series show a hydrogen content of 12.5, 12.5, 12.6, and 11.8%, respectively, with a weighted mean

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Table 1. Contribution of raw wool components to PMR signal (6 g sample) [a]

Component	Percent (by wt)[b]	Amt, g	Signal/g from PMR measurement	Contribution to raw wool PMR signal
Wool protein	55	3.30	0.3	1.0
Suint	7	0.42	0.5	0.2
Vegetable matter, dirt	7	0.42	0.3	0.1
Wax	18	1.08	30.0	32.4
Water	13	0.78	Eliminated by drying	0

[a] Blank (wool protein, suint, vegetable matter, dirt): mean 1.2, SE 0.14, $n = 20$.
[b] Typical values from Prospect Laboratory, and consistent with Onions (6) and Howitt and Preston (7).

of 12.4%, and with very small changes in hydrogen percentage throughout the likely range in each series.

This constancy of hydrogen content indicated that hydrogen response would be both more meaningful and more fundamental as a measure of wax than is extracted weight.

However, wool wax, mp 40–45°C (5), is a solid at ambient temperatures and PMR normally requires liquids or solutions. Wool wax also contains hydrogen in the fiber protein, in suint, in water, and in the contaminants (Table 1). Thus, in addition to the requirements mentioned above, it was necessary to address the following problems: Could the detection sensitivity for hydrogen in wax be increased significantly by increasing the temperature at which the sample was measured, and if so what was the optimum temperature? Could hydrogen in wax be determined in the presence of other sample hydrogen, and if so, how could this be done optimally? Was the inter- and intra-breed wax composition so variable as to vitiate the method? How was the PMR method correlated with the routinely used Soxhlet extraction?

These investigations were carried out in conjunction with an assessment of the wide-line PMR unit itself for conformity to specification.

Assessment of PMR Method

Materials

(a) *Instrumentation.*—(1) *Wide-line PMR.*—Newport MK III operating at a radiofrequency of 2.7 MHz with a permanent magnet field of 635 gauss. The unit was equipped with a temperature controller (−20°C to +90°C) and, in our laboratory, was interfaced to an STC Model 33 teletype for readout. Automatic reset and reread were provided via the teletype interface. An inbuilt 7 cm cathode ray oscilloscope enabled the PMR absorption signal to be observed and tuned.

The 40 mL probe assembly (power 40 milligauss peak to peak, nominal sensitivity 15 mg oil) was used for the study reported in this paper because the larger volume assembly 150 mL (power 20 milligauss peak to peak, nominal sensitivity 50 mg oil) appeared to offer no advantages in our work.

(2) *High resolution PMR.*—Hitachi Model R-24A (14.092 kilogauss, 60 MHz); range 0–10 ppm.

(3) *Infrared spectrophotometer.*—Perkin-Elmer Model 377 (range 4000–400 cm^{-1}).

(b) *Wool samples.*—Raw wool samples were collected from Merino and Merino-crossbred field fleeces. Wax was determined on subsamples by both wide-line PMR and Soxhlet extraction (see below). In addition, selected samples were processed to yield wax, suint (8), vegetable matter, and clean wool fiber which were used to assess their contribution to the wax PMR signal.

Experimentation and Results

(a) *Soxhlet extraction procedure.*—Wool samples (5 g dry weight) were placed in Soxhlet thimbles and extracted 3 h with 130 mL petroleum ether fraction (Shell X-222, bp range 40–95°C). The bulk of the solvent was siphoned from the Soxhlet thimble and the remainder was allowed to boil off. The flask containing the wax was dried overnight at 105°C; the wax was weighed and its percentage in wool was calculated. Blank correction (solvent and thimbles) was negligible.

(b) *Wax composition.*—To investigate uniformity of wax composition from different fleeces and breeds, without which the PMR method is not viable, samples of Soxhlet-extracted wax were analyzed on a Perkin-Elmer infrared spec-

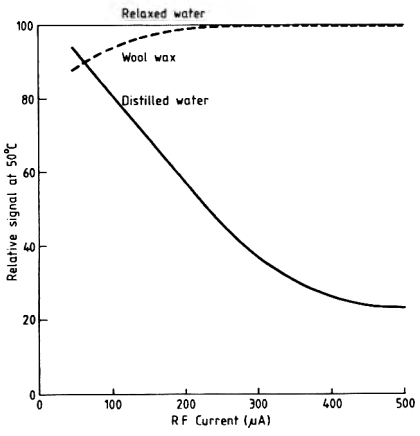

Figure 1. Check on signal saturation at 50°C for wool wax (2 g), using 40 mL sample assembly. Signal from samples is function of RF current (μA) at constant AF gain (1000). Relaxed water is adjusted to 100% for each RF current. Results show that, under chosen operating conditions, signal is not saturated.

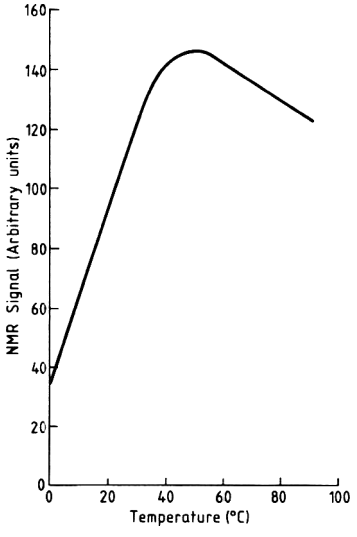

Figure 2. PMR signal as function of temperature from 5 g wool wax sample in 40 mL assembly over temperature range 0-90°C; RF current 500 μA, AF gain 1000, integration time 128 s.

trophotometer between sodium chloride plates, and on a high resolution PMR instrument in deuterochloroform. No differences were detected between spectra.

To check for thermal degradation, wax from the Soxhlet procedure was compared with cold-extracted wax from which the solvent had been removed by rotary evaporation: No differences were detected by either infrared or high resolution PMR spectroscopy.

As a further check on the uniformity of wax composition, hydrogen was determined by microanalysis (AMDEL Laboratories, Melbourne, Australia) on 4 randomly chosen samples. Results, with PMR signal per g in brackets, are as follows: 11.43% (28.6); 11.88% (29.5); 11.70% (29.3); 11.94% (28.1); mean value, 11.74% (28.9). These values are in agreement with Motiuk's figures for the composition of wool wax previously cited (3, 4).

Wide-Line Measurements

(a) *Determination of operating parameters.*—The initial evaluation of the instrumental method entailed the determination of optimal values for audiofrequency (AF) gain and radiofrequency (RF) current, and probe operating temperature. For these optimizations, pooled, solvent-free wool wax from Soxhlet extractions with Shell X-222 was used. The 3 variables were optimized iteratively for 1, 3, and 5 g wax samples.

For all samples, the maximum signal was obtained for maximum AF gain (setting 1000) and maximum RF current (500 μA); because this did not lead to signal saturation ((9) and Figure 1), these settings were used throughout.

The signal vs temperature curve (Figure 2) shows that the optimum temperature is in the region of 50°C, with only a 3% variation in signal from 40 to 60°C; 50°C was selected as the operating temperature in our experimentation. Instrument temperature was controllable to better than 0.5°C, and temperature stability was thus not a significant source of error.

Using the above established instrumental settings, the PMR method was calibrated and then investigated for sensitivity and precision.

(b) *Calibration.*—The instrument was calibrated using the pooled wax sealed in 20 mL glass ampules under nitrogen to prevent oxidation and long-term loss of any volatiles. Weights used were 0.00, 0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 3.00, and 5.00 g.

As expected for an NMR signal, without saturation, the instrument was linear over the entire range from 0.00 to 5.00 g wax, i.e., from 0 to 100% wax in 5 g wool on a dry basis.

Statistical treatment of the calibration data yields an equation for the linear regression of y on x of:

$$y = 29.771(\text{SE } 0.030)x$$

where y = PMR signal, and x = wax weight, with $r^2 = 0.99$; and $n = 8$. The PMR signal per g wax is thus 29.8 units.

Table 2. Determination of precision of NMR determination of wax in wool [a]

Statistic	Integration time, seconds			
	1	8	32	128
Mean PMR signal	59.3	59.4	59.4	59.4
Range	54.4–63.9	57.3–61.0	58.6–60.4	59.0–59.7
Standard deviation	1.95	0.67	0.28	0.15
Standard error	0.19	0.07	0.03	0.02

[a] 2 g wool wax standard; 100 measurements at each integration time.

(c) *Sensitivity.*—The sensitivity of the 40 mL probe assembly as calculated from the calibration graph was 17 mg wax for an output signal change of 0.5 unit (see calibration statistics).

The calculated sensitivity was checked by adding known small amounts of wool wax (approximately 17 mg) to the probe with the instrument conditions as above, and an integration time of 128 s. (a) Wool wax (16.0 mg) was added to dry, raw wool of 5 and 20% wax content; the output signal increase was measured. (b) Wool wax (16.7 mg) was added to the empty sample probe, and the output signal increase noted. Statistical treatment of the data yielded the following results: For 5% wax, PMR signal increased 0.5 ± 0.05 (SE), (n = 20); for 20% wax, PMR signal increased 0.5 ± 0.04 (SE), (n = 20); for empty tube, PMR signal increased 0.4 ± 0.08 (SE), (n = 10). Within the precision of the instrument, both (a) and (b) represent 100% recovery of the added wax.

On a 5 g dry wool sample, 15 mg represents a sensitivity of 0.3% wax. Since the 40 mL probe will hold up to a maximum of 10 g wool in its sensitive volume, the instrument sensitivity can be increased to 0.15% wax, thus enabling determination of residual wax in, for example, scoured wool (approximately 1–2% residual wax). Because scouring removes suint, no background correction for this is required for such samples.

In our work the major interest was raw wool with wax in the region 5–30% for a 5 g dry sample which contains 250–1500 mg wax. Instrument sensitivity was thus more than adequate.

(d) *Precision.*—Precision was investigated by repeated determination (n = 100) on the 2 g calibration sample using separately, each of the 4 integration times (1, 8, 32, 128 s). Results are presented in Table 2.

To illustrate, based on the standard deviation for the 128 s integration time (Table 2), 95% of the random PMR values would fall between 59.36 and 59.44 for a 2 g wax sample. That is, a raw wool with 40% wax would be measured with a precision of 0.06%.

Contribution of Non-Wax Wool Components to PMR Signal

The PMR response to weighed amounts of dry suint, vegetable matter, and wool fiber (protein) was determined in the same manner and under the same conditions as for wax. At 50°C, the PMR signal of raw wool has a narrow peak about 0.1 gauss arising from mobile components such as liquid wax and water, superimposed on a broad hump (10 gauss) due to solid components such as suint, wool fiber (protein), and vegetable matter, which are in some form of crystal lattice. The problem we have, in effect, been investigating, is how to maximize by experiment the contribution of the narrow (wax) peak, to which the instrument is tuned, to the background peak.

Water (11.2% H) contributes the same signal per gram as wax, and the wide-line PMR unit cannot distinguish the water and wax signals. Water had to be removed by heating for 16 h at 105°C. Vacuum- and freeze-drying were also effective but less convenient.

Water and wax signals are, in principle, distinguishable by their relaxation times in pulse PMR, but our initial investigations indicated this method was not viable because of considerable (unsolved) problems in signal processing.

Wax could be rendered liquid by heating, and the maximum signal was obtained at 50°C (Figure 2). The contribution, at 50°C, of the non-wax components to the wax signal is shown in Table 1. Here the blank correction is about 5% of the total signal, and numerous experiments showed it to be substantially constant: A correction for background is thus valid.

Recovery of Wax Added to Wool Fiber

To ensure that wax distribution was not critical within the sensitive volume of the PMR probe,

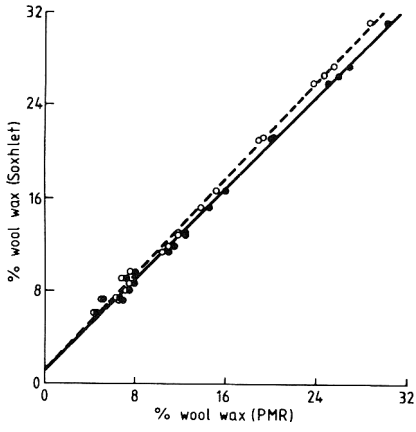

Figure 3. Comparison of wide-line PMR and Soxhlet estimation of wax in raw wool.

In Method 1 (●—●), wax was obtained from calibration graph. In Method 2 (O- - -O), points were plotted for individual wax signal values. Linear regression of Soxhlet wax (y) against PMR wax (x) was:

$$\text{Method 1.} \quad y_1 = 1.0257(\text{SE } 0.2277) + 0.9894(\text{SE } 0.0142)x_1$$

$$\text{Method 2.} \quad y_2 = 1.0167(\text{SE } 0.2249) + 1.0432(\text{SE } 0.0148)x_2$$

In both cases $r^2 = 0.99$ and $n = 20$.

pooled wax in known amounts was added to clean wax and suint-free wool fiber and also to dry raw wool as uniformly as possible; and as non-uniformly as possible. In both cases the recovery of wax was 100%, i.e., thus there was no difference in the signal/g of wax. Thus, it is valid to use sealed calibration standards in which the wax distribution is not the same as in wool.

These results indicated that the wax response was not position-dependent within the sensitive volume of the probe and thus that samples of various sizes (up to the maximum of 10 g) could be processed with constant sensitivity: Experiment confirmed this.

Standard PMR Measurement Procedure

As the outcome of the investigation reported in this paper, a standard PMR measurement procedure was developed.

Raw wool samples (approximately 6 g) were weighed into glass vials and dried overnight in an oven at 105°C. After reweighing (if dry weight was required), vials were transferred to a 50°C oven (for pre-measurement conditioning) and then to the 40 mL probe assembly of the NMR unit set at 50°C; as before, the AF gain was 1000, and the RF power was 500 μA. Each sample was measured in triplicate with an integration time of 128 s. After the background correction (calculated from Table 1 for each batch) was subtracted, the weight of wax corresponding to the NMR signal was read from the calibration graph, and the percentage of wax in the dried raw wool sample was calculated.

Results

Comparison of PMR and Soxhlet Methods

Since wax is, at present, routinely measured by Soxhlet extraction, a comparison between the results obtained by this method and those obtained by wide-line PMR is important. Duplicate raw wool samples from 10 fleeces were measured by the nondestructive PMR technique using the standard procedure previously described in this paper. Then the same samples were processed by the standard Soxhlet procedure. The output signal from the PMR was converted to a wax weight by 2 methods.

Method 1: Wax weight was read from the calibration graph. Results are shown in Figure 3. Statistical treatment of the data yields a linear regression for Soxhlet wax (y_1) vs PMR wax (x_1) of $y_1 = 1.0257$ (SE 0.2277) + 0.9894 (SE 0.0142)x_1 with $r^2 = 0.99$; and $n = 20$.

Method 2: Here, the signal per gram of *each* of the Soxhlet-extracted waxes was determined by PMR. The relevant value was used to calculate wax weight from the previously determined PMR signal for each raw wool sample.

Method 2 was carried out to further check for any difference in wax hydrogen composition. As before, no gross differences were found. The mean signal per gram for the 20 samples was 31.4 (range 29.0–32.8) with a standard deviation of 0.97 and a standard error of 0.22. The results are also shown in Figure 3 which thus constitutes a direct comparison of the 2 methods.

Statistical treatment of the data from Method 2 gives a linear regression for Soxhlet wax (y_2) vs PMR wax (x_2) of $y_2 = 1.0167(\text{SE } 0.2249) + 1.0432(\text{SE } 0.0148)x_2$, with $r^2 = 0.99$; and $n = 20$.

Statistical treatment (comparison of slopes) shows the lines in Figure 3 are significantly different ($P < 0.05$).

However, to illustrate what this means in practice, the 2 curves were compared as predictors of wax amount, using, in each case, a PMR value of 20% wax. Method 1 predicts a Soxhlet wax of 20.81%, with SE of 0.51, and 95% confidence interval of (19.73, 21.89); Method 2 predicts

a Soxhlet wax of 21.88% with SE of 0.51, and 95% confidence interval of (20.81, 22.95).

On the predicted values this represents a precision of about 5%, and since, as the next section shows, intra-fleece variations are likely to be considerably more than this, the single calibration graph (Method 1) suffices for all except the most precise work.

Discussion

The PMR technique meets the requirements specified in that it involves minimal sample preparation (only drying is required), and operation of the instrument is simple and largely automatic. Total operator time (weighing, drying, and subsequent transfer to PMR unit) is estimated at less than 2 min per sample. Instrument time per sample (duplicate reading with 128 s integration time) is about 5 min and one operator could therefore process in excess of 90 pre-dried samples per 8 h day. Instrument time can be reduced considerably by using a shorter integration time (32, 8, 1 s), thus enabling a daily throughput of several hundred samples, but with decreased precision (Table 2). Automation of the process is feasible; the major problem is the maintenance of a 50°C environment for the sample changer.

Because operator involvement is minimal, the sensitivity and precision of the PMR method is, in our experience, superior to the Soxhlet procedure in routine use.

Our results indicate that wax does have a variable composition but this variability did not prove to be the problem initially anticipated. Nevertheless, the PMR method is empirical and must be used with an awareness of the assumptions underlying it.

Under marginal conditions (small sample size and/or a low wax percentage), the highest precision can be obtained for the PMR method by basing (where feasible) the calibration on wax extracted from similar samples to those under study.

The blank correction for suint, vegetable matter, and wool fiber should be similarly determined. If the conductivity method recently reported by Lipson and Hilton (8) proves viable, wax and suint could be determined sequentially and a direct correction, rather than a calculated one, could be applied.

Wools heavily contaminated with vegetable matter may present a high blank problem. However, Townend and Russell (10) report that 85% of the Australian wool clip has less than 7% vegetable matter, and thus would present minimal problems to the PMR method. It has been our experience that the factor limiting the accuracy of wax determination by any method is the intra-fleece variability.

The PMR method has been successfully used in our laboratories for 3 years to measure samples for research projects. Wax in raw wool from a variety of field stations has also been measured: In all cases, results have been satisfactory. The long- and short-term stability of the PMR instrument is good.

Conclusion

The wide-line PMR technique is a replacement for the Soxhlet method for the determination of wax in wool and has the advantages detailed in this paper. It is especially applicable where large numbers of samples need to be screened rapidly, e.g., in fleece-rot studies, in wool evaluations, and in wool-scouring plants.

It would seem to have a place in the objective measurement of the properties of wool in which scheme in excess of 4 million bales were reported in the Australian Wool Corporation Interim Annual Report, as being core sampled in 1979–1980 (11). Although, in our view, one is urgently needed, no instrumental method for the determination of the other components of raw wool exists at present.

Acknowledgments

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References

(1) Robertson, J. A., & Morrison, W. H. (1979) *J. Am. Oil Chem. Soc.* **56**, 961–964
(2) Ford, P. T., Friswell, N. J., & Richmond, I. J. (1977) *Anal. Chem.* **49**, 594–596
(3) Motiuk, K. (1979) *J. Am. Oil Chem. Soc.* **56**, 651–658
(4) Motiuk, K. (1979) *J. Am. Oil Chem. Soc.* **56**, 91–97
(5) von Bergen, W. (1963) *Wool Handbook*, Vol. 1, 3rd Ed., Interscience Publishers, New York, NY, p. 214
(6) Onions, W. J. (1962) *Wool, An Introduction to Its Properties, Varieties, Uses, and Production*, Interscience Publishers, New York, NY, p. 118
(7) Howitt, F. O., & Preston, R. (1960) *J. Text. Ind.* **51**, T841–T856
(8) Lipson, M., & Hilton, R. A. (1981) *Text. Inst. Ind.* **19**(6), 182–183
(9) *Newport Analyzer MK III User Handbook*, Section 5, p. 26–28
(10) Townend, P. P., & Russell, K.P. (1980) *Wool Sci. Rev.* **56**, 46
(11) *Interim Annual Report* (1979–80) Australian Wool Corp., p. 38

TECHNICAL COMMUNICATIONS

Polarographic Determination of Kjeldahl Nitrogen

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A simple and rapid method is described for determining ammonia content of a Kjeldahl digest by polarography. The digest is diluted and pipetted into a solution of formaldehyde in an acetate buffer. The hexamethylenetetramine produced by reaction between ammonia and formaldehyde is determined electrochemically at −1.04 V. Three different samples (milk, bread, and cashews) were analyzed and Kjeldahl nitrogen showed an average relative standard deviation of 3.4%; repetitive analyses of the same digest demonstrated a relative standard deviation of 1.5%.

There are a number of methods for quantitative determination of total protein (1). Of these, the Kjeldahl procedure (2) is routinely used. The Kjeldahl method for total protein analysis involves 3 steps: catalyzed digestion of the sample with boiling sulfuric acid to reduce all proteinaceous nitrogen to ammonium sulfate, isolation of ammonia by steam distillation of the alkaline digest into an acidic trapping solution, and quantitation of ammonia by back-titration of the acid trap with dilute acid. Percentage nitrogen determined is converted to percent protein by a factor which depends on the nature of the digested foodstuff (1). Attempts have been made to determine the ammonia present after digestion by using an ammonia electrode (3). Polarography (4) has been used to determine the ammonia after the distillation step, but it is neither more precise nor more rapid than titration. This study demonstrates the use of polarography for direct determination of ammonium in the acidic digest.

METHOD

Reagents

Reagents were ACS grade and were used without further purification.

(a) *Mercury catalysis tablets.*—Containing 1 g sodium sulfate and the equivalent of 0.1 g mercury (BDH Chemicals Ltd, Poole, UK).

(b) *Ammonium chloride.*—99.5% (Analar grade, BDH Chemicals); dried over silica gel overnight before use. *Standard solution:* Accurately weigh 314.5 mg into 100 mL volumetric flask, add 50 mL double-distilled water and 2 mL concentrated sulfuric acid, and dilute to volume with double-distilled water. Dilute fresh daily for use in standard addition procedure: Pipet 10 mL standard into 50 mL volumetric flask and dilute to volume with double-distilled water (200 ppm ammonia).

(c) *Supporting electrolyte.*—Weigh 10.4 g sodium acetate, dissolve in water, add 32 mL glacial acetic acid, dilute to 200 mL, and add 200 mL 37% formaldehyde (1:1 mixture of 0.4M acetate buffer and 37% formaldehyde, pH 4.5).

Apparatus

Polarographic measurements were made with Model 174A polarographic analyzer, Model 303 static mercury drop electrode, and Model RE0074 X-Y recorder (Princeton Applied Research, PO Box 2565, Princeton, NJ 08540). For differential pulse operation: drop time 0.5 s, pulse height 25 mV, scan rate 5 mV/s, initial potential −0.7V, final potential −1.2V, and current sensitivity 100 nA/in. Three-electrode operation was used with dropping mercury electrode, silver/silver chloride reference electrode, and platinum auxiliary electrode. Glass polarographic cell, containing 10 mL electrolyte, was purged 4 min before use with stream of nitrogen which had been deoxygenated by bubbling through vanadous chloride solution over amalgamated zinc.

Preparation of Sample

Digest from any size Kjeldahl flask may be used; adjust with double-distilled water to give ammonia content of 0.5–20 mg/mL. In this study: Accurately weigh 200 mg sample into 100 mL Kjeldahl flask, add 4 mL sulfuric acid (sp. gr. 1.84), 1 mercury catalyst tablet, and 1 bumping chip. After digestion is complete (1½ h), quantitatively transfer contents of Kjeldahl flask to 100 mL volumetric flask with aid of 3 washings of double-distilled water and dilute to volume with double-distilled water.

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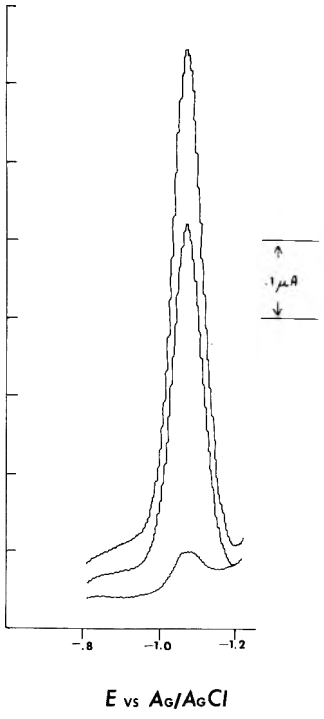

Figure 1. Typical differential pulse polarograms of blank cell, sample from a Kjeldahl digest, and sample after standard addition.

Determination

Scan supporting electrolyte through voltage range, reset initial voltage and recorder, pipet either 50, 100, or 250 μL diluted sample digest into the cell (chosen aliquot size depends on percent protein in sample, corresponding to ranges of >50%, 25-50%, and <20%, respectively), purge 2 min, wait 15 s after termination of purge, and scan sample through voltage range. Reset voltage and recorder, pipet 50 μL standard solution into cell, purge 2 min, wait 15 s, and scan.

Calculations

Determine concentration of nitrogen in sample from equation:

Ammonia, % = $[(P - P_b)/(P' - P)] \times R/100$

where P, P_b and P' = peak height of sample, blank, and sample plus standard; $R = (C' \times V')/(W \times V)$, where C' = concentration of standard in μg/mL; V' = volume of standard added to cell in μL; W = weight of sample in mg; and V = volume of sample added to cell in μL. The % protein can be determined by multiplying % nitrogen by appropriate factor for sample type being analyzed.

Table 1. Determination of protein (%) in 3 commercial foodstuffs [a]

Detn	Cashews	Nonfat powdered milk	Arabic bread
1	21.7	33.6	9.0
2	21.3	31.2	8.8
3	22.9	34.4	9.4
4	22.6	33.9	9.5
5	22.5	33.1	9.5
Av.	22.2	33.2	9.2
SD	0.67	1.23	0.32
Rel. SD, %	3.0	3.7	3.5

[a] Five replicate determinations.

Results and Discussion

In dilute aqueous solutions, formaldehyde combines with ammonia to yield hexamethylenetetramine in accordance with the equation

$$4NH_3 + 6HCHO \rightarrow (CH_2)_6N_4 + 6H_2O$$

The reaction occurs quantitatively and forms the basis of a titrimetric method for determining formaldehyde (5). In the present study, ammonia in the digest reacts with formaldehyde in acetate buffer to give hexamethylenetetramine which is determined at −1.04 V vs Ag/AgCl reference electrode by differential pulse polarography. A typical polarogram is shown in Figure 1. The reaction between ammonia and formaldehyde in the supporting electrolyte used in this study required approximately 80 s for quantitative results. The linearity of current vs ammonia concentration was checked by pipetting into the polarographic cell successive 10 μL (2 μg NH_3) aliquots of standard ammonia solution ranging from 0 to 24 μg (2400 ppb in the cell) (2 μg of ammonia is equivalent to a protein level of 2.1% by the method in this paper). A linear regression of the amount pipetted against the observed detector current response gave a correlation coefficient of 0.996. The blank cell showed a positive response of 152 ppb for ammonia. The formaldehyde was suspected as its source. Despite this positive cell blank it was possible to determine ammonia concentrations to 50 ppb. This represents a protein level of 0.5% in the original sample; the detectable level for protein itself can be taken lower than this by pipetting in larger aliquots of the diluted sample digest. The sample digestion and dilution methodology is designed to give a final concentration level of ammonia in the range of 200–2000 ppb.

Three different foodstuffs were analyzed, and the results are shown in Table 1; no interference

from other ingredients that survive the digestion are evident. One of the replicates from each run was analyzed 4 times; the average relative standard deviation for these 3 samples was 1.5%. Comparison of the relative standard deviations shown in Table 1 with relative standard deviations (average on 4 samples = 4.0%) published for an AOAC collaborative study (6) partly concerned with the traditional Kjeldahl procedure shows that the polarographic technique is more precise. This polarographic method reduces both the amount of manual manipulation and reagent preparation required in the classical procedure and, although polarography offers an expensive alternative to distillation and titration, it can be used for a variety of other analyses as well.

REFERENCES

(1) Pearson, D. (1977) *The Chemical Analysis of Foods*, Sentry Press, New York, NY, p. 9
(2) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **2.055-2.056**
(3) *Analytical Methods Guide* (1978) Orion Research, Cambridge, MA, pp. 35-37
(4) *Application Brief A-3* (1976) Princeton Applied Research, Princeton, NJ
(5) Bradstreet, R. B. (1940) *Chem. Rev.* **27**, 331-350
(6) Malanoski, A. J. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 345-347

Improved Radioimmunoassay of Staphylococcal Enterotoxin A

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A simple solid-phase radioimmunoassay was developed for detecting staphylococcal enterotoxin A (SEA) in food. The method detected 85-100% SEA added to extracts of 6 kinds of foods, and 52-100% SEA added directly to foods at a concentration of 1 ng/g. Assay sensitivity is about 0.3 ng toxin/mL extract. The method is specific for SEA; results are only marginally affected by staphylococcal enterotoxins B and C_2 present at concentrations as high as 500 ng/mL extract.

Staphylococcal enterotoxin A (SEA) belongs to a group of closely related simple proteins that are causative agents of staphylococcal food poisoning. Sufficient partially purified staphylococcal enterotoxins have been available for use in the microslide test, which is the method most frequently used to detect enterotoxin in food (1). The availability of highly purified enterotoxins has made it possible to develop radioimmunoassay systems requiring relatively simple sample preparation and able to detect as little as 1 ng enterotoxin/g food in food extracts (2). This paper describes some results and characteristics in the application of a solid-phase radioimmunoassay (RIA) of SEA and indicates that it may meet these requirements. It is a modification of our initially reported assay (3), using different incubation conditions and a better defined tracer. The present assay is about 10 times more sensitive than our earlier assay. The procedure has been used in our laboratory for quantitation of enterotoxin in a variety of food preparations. Presence of large amounts of organic matter in food extract samples did not appear to interfere with the RIA test. This finding enabled the use of one buffer for the standard curve to determine the concentration of toxin for a variety of food items. The alternative is to employ food extract from a comparable, toxin-free food source as a negative control instead of buffer (4).

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Experimental

Reagents and Apparatus

(a) *Buffer A.*—0.1M carbonate-bicarbonate buffer (pH 9.6) containing 0.05% (w/v) sodium azide.

(b) *Buffer B.*—0.5% (w/v) bovine serum albumin (RIA grade, Sigma) dissolved in 0.05M

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phosphate buffer (pH 7.4)–0.15M sodium chloride containing 0.05% (w/v) sodium azide.

(c) *$Na^{125}I$.*—Atomic Energy of Canada, Ltd, Ottawa, Ontario, Canada.

(d) *SEA standards.*—Prepare doubling dilutions of standard SEA in buffer B to give 5 working standard solutions in range 0.078–1.25 ng/100 μL. Standard toxin used in this study was obtained from M. S. Bergdoll.

(e) *Chromatographic column.*—K 26/40, Pharmacia Fine Chemicals AB, Uppsala, Sweden.

(f) *Assay tubes.*—Polypropylene test tubes, 12 × 75 mm. (No. 9950, Canada Wide Scientific Ltd, Ottawa, Ontario, Canada).

(g) *Gamma counter.*—Beckman 4000 automatic with scintillation γ-counter.

Preparation of ^{125}I-SEA

Labeled SEA was prepared by a modification of the method of Freedlender et al. (5): To 15 μL solution containing 1 mg pure SEA/mL water, add 2.5 mL 0.05M sodium phosphate buffer, pH 7.4, and ca 10 μL solution containing 1 mCi ^{125}I. Dropwise and with continuous stirring, add 0.5 mL freshly prepared solution of chloramine T (30 μg/mL) in 0.05M phosphate buffer. After 10 min, dropwise add 0.7 mL freshly prepared solution of sodium metabisulfite (30 μg/mL) in 0.05M phosphate buffer and continue stirring additional 10 min. To remove unreacted iodide, apply reaction mixture to 3 mL column of Dowex-1 resin which has been equilibrated with 100 mL 0.05M phosphate buffer and treated with 2 mL 2% (w/v) bovine serum albumin (6). Collect 0.5 mL aliquots and pool fractions 2–8 which contain labeled SEA.

Use gel filtration for assessing degree of iodination and for determining extent of damage or degradation of ^{125}I-SEA: Apply 2 mL freshly labeled SEA to 2.6 × 33 cm column of Sephadex G-100 equilibrated in buffer B at room temperature. Elute with buffer B and collect about 60 fractions of 4.0 mL at 10–15 mL/h. Count fifty 10 μL aliquots in γ-counter 1 min.

For most SEA iodinations, an amount of high molecular weight radioactive material appears shortly after void volume on Sephadex column (peak I of Figure 1). This material represents mainly aggregated or damaged enterotoxin which only poorly binds to the antibody. Fractions from Peak II, corresponding to purified enterotoxin, are selected to minimize contamination with material from peaks I and III, the latter being mainly free ^{125}I. Selected fractions are pooled and stored at 4°C. Iodination efficiency is calculated from elution pattern. Labeled enterotoxin is usable for up to 2 months, after which assay tends to become unstable, resulting in erratic replicates and reduced dose response.

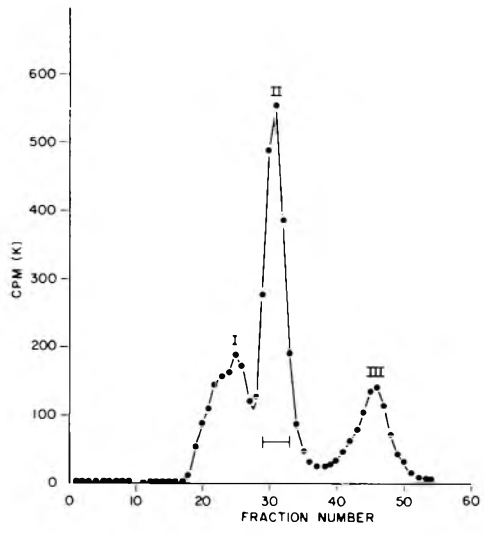

Figure 1. Pattern of elution of radioactivity after gel filtration (Sephadex G-100) of ^{125}I-SEA. Fractions (4.0 mL) were pooled as indicated. About 10% total radioactivity was incorporated into peak II.

Extraction Procedure

Use method of Robern et al. (7): Blend solid food samples with 2 volumes of buffer B, and centrifuge at 4°C for 30 min at 34 900 × g. Adjust supernate to pH 4.6 with 2N HCl, let stand at room temperature 10 min, and then centrifuge as above. Re-adjust to pH 7.4 with 2N NaOH, store at 4°C for 2 h, and centrifuge once more.

Assay Procedure

Salient features of the radioimmunoassay are as follows: Antibody-coated plastic tubes are incubated with known amounts of SEA or test sample at 4°C. Tube contents are removed, labeled SEA is added to each tube, and incubation is continued briefly at room temperature. Antibody bound and free label are separated by aspiration of tubes.

(a) *Preparation of antibody-coated tubes.*—Coating procedure follows principles of Catt and Tregear (8), who elaborated in detail the requirements for coating plastic tubes with rabbit gamma globulin. Tubes were coated at room temperature. A range of antibody dilutions prepared in buffer A were used and coating time was 10 min. Antiserum against SEA (anti-SEA)

was obtained by immunization of rabbits with SEA (9). At the antibody dilution where 25–30% of the tracer (15 000 cpm) was bound in the absence of unlabeled SEA (corresponding to our assay conditions), 0.078 ng unlabeled SEA displaced about 15% bound tracer from solid-phase antibody. This dilution of antibody, usually 2 000- to 3 000-fold, was used for preparation of standard curves.

Dispense 0.2 mL rabbit anti-SEA antibody in buffer A to polypropylene test tubes. Let tubes remain at room temperature 10 min. Remove antibody solution by decantation and wash tubes twice with 1 mL buffer B by vortex mixing for a few seconds. Then add 1 mL buffer B to each tube and let tubes remain at room temperature 30 min. Remove buffer as completely as possible by decanting and draining tubes. Use tubes for assay within 1 h.

(b) *RIA procedure.*—Add 100 μL standard SEA or unknown to coated tubes. Add 100 μL buffer B to each tube to give final volume of 200 μL. Shake mixture, and incubate 20 h at 4°C. Assay each standard and unknown in duplicate, zero dose tubes in triplicate. After this pre-incubation, discard contents and wash tubes twice with 1 mL ice-cold buffer B on vortex mixer; invert tubes for complete drainage. Then add 200 μL (15 000 cpm) ^{125}I-SEA to each tube. Let tubes remain at room temperature 2 h. Then remove contents by aspiration and wash each tube twice with ice-cold 0.85% (w/v) sodium chloride. Count each incubation tube (antibody-bound ^{125}I-SEA) in γ-counter 5 min. Express binding as ratio of bound to total counts (B/T) after making correction (150 cpm, in the present case) for background radioactivity.

Results and Discussion

The standard curve was drawn manually by plotting the ratio of bound counts to total counts (B/T) on the vertical axis against the arithmetic dose of enterotoxin on the horizontal axis. Figure 2 shows a typical standard curve of the system described. The advantage of this approach is that it presents a reasonably complete picture of the characteristics of any one assay run. For instance, the amount of labeled enterotoxin bound in the zero dose tubes can be read directly from the curve; if this is low, it suggests deterioration in either the labeled enterotoxin or the antibody. Unknown values can be obtained by interpolation on the standard curve.

Standard curves were established on 12 days with 2 lots of labeled SEA. Mean values and standard deviations of the percent bound counts

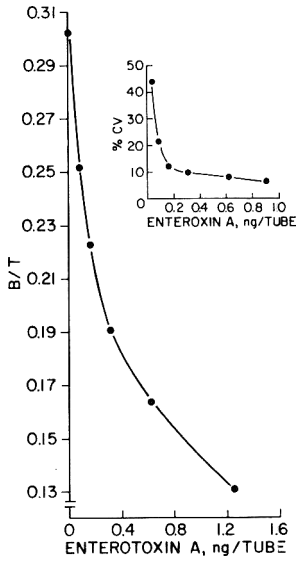

Figure 2. Enterotoxin A standard curve. Inset: predicted intra-assay precision.

($B/B_0 \times 100$) were calculated as 84.0 ± 4.2, 73.4 ± 4.0, 64.8 ± 2.8, 53.5 ± 3.4 and 40.7 ± 4.3 for added SEA/tube of 0.078, 0.156, 0.312, 0.625, and 1.25 ng.

Intra-assay precision of the method was determined by calculating the variance of replicate responses for several doses on the standard curve and describing the relationship between the variance and mean of the dose-response (B/T) by regression analysis, as suggested by Rodbard et al. (10). The power function (their Equation 5) was fit to the data without weighting and by the use of a hand calculator. The standard deviation of the SEA estimate at any point on the standard curve was estimated from the standard deviation of the response variable and the slope of the dose-response curve calculated at this point (11). Precision, expressed as the predicted coefficient of variation of the dose, varies with the part of the dose-response covered (Figure 2, inset). It is less at the extreme of low SEA concentrations. In the region of the standard curve corresponding to 30–65% inhibition of binding, precision of duplicate samples is about 10%.

In this assay, the mean of observed B/T values in the absence of unlabeled SEA could usually be distinguished statistically ($P < 0.05$, one-tailed t-test (12)) from the response in the presence of an unknown, assayed in duplicate, if 6% of the maximum binding is displaced. In an incubation volume of 0.2 mL, this displacement corresponds to about 30 pg SEA, as estimated by dose

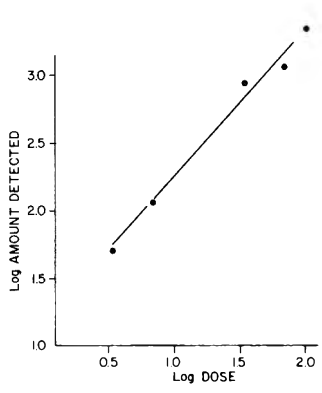

Figure 3. Testing parallelism of standard and unknown (strain S-6 culture fluid) dose-response curves, using simple linear regression. Log (pg SEA detected) vs log sample volume (10^{-2} μL).

interpolation on the standard curve. This gives a working sensitivity for the assay of 0.3 ng/mL with 100 μL as the largest sample size.

Specificity of the antiserum was assessed by comparing the ability of other related proteins such as staphylococcal enterotoxins B and C_2 at various concentrations to compete with ^{125}I-SEA for binding sites on the antiserum. When staphylococcal enterotoxin B (Makor Chemicals, Jerusalem) and C_2 (13) were added at 50 ng/tube (equivalent to 500 ng/mL extract), about 10% of labeled SEA was displaced from the antibody with enterotoxin B, and 6% with enterotoxin C_2. Increasing the concentration of each of these enterotoxins to 200 ng/tube did not increase the percentage of labeled SEA displaced from the antibody with enterotoxin B, but resulted in further displacement with enterotoxin C_2 (to 15%). This effect is a reflection of the specificity of the antiserum and may be different for other antisera.

An important criterion for specificity is a linear decrease in measured immunoreactive SEA levels with dilution. This was demonstrated with the supernatant fluid of cultures of *S. aureus* S-6, a strain producing both enterotoxins A and B. Dilutions were prepared to encompass the pg dose range of the assay. Samples were assayed in duplicate, and the apparent amount of material detected per tube was calculated. Analysis of an experiment, following Rodbard et al. (14), used the $\log_{10}$ transformation of all data to correct for heterogeneity of variance. These data are shown in Figure 3. Calculation of a straight line by means of simple least squares regression analysis gave a slope of 1.09 and a correlation of 0.992, which indicates that good parallelism was achieved.

Accuracy was determined by recovery studies of SEA added to food extracts containing less than 0.3 ng endogenous SEA/mL (determined by radioimmunoassay). Samples were extracted by the acid-base method of Robern et al. (7). When oily specimens were examined, the final supernatant fluid was filtered through a Millex filter unit (0.45 μm, Millipore) and the filtrate was used for analysis. SEA was added to the extract at 12.5 ng/mL and at halving dilutions of 6.25, 3.12, and 1.56 ng/mL. Buffer B was the diluent and 100 μL portions were assayed in duplicate at each dilution. The results are summarized in Table 1. The average recovery (about 94%) was determined from the slope of the straight line ($y = 0.936x - 0.0127$; $r = 0.987$), calculated by least squares regression analysis of the amount of SEA detected vs amount of SEA added, plotted for all data on rectangular coordinates. Variation between duplicates was no greater than 15%.

Unknown samples are regularly tested at multiple dilutions and a mean SEA concentration is calculated. Parallelism between the dilutions of food extract and SEA standards provides evi-

Table 1. Recovery of staphylococcal enterotoxin A added to extracts of some foods

Food	Slope[a]	Intercept	*r*
Cheese	0.914	−10.1	0.998
Ham	0.925	3.4	0.978
Pasta	0.864	−29.6	0.999
Salmon	0.925	62.3	0.994
Salami	1.022	−76.0	0.990
Sardine	0.850	12.0	0.989

[a] SEA (pg/tube) vs SEA added (pg/tube) was analyzed by regression which permitted straight lines and *r* values to be calculated.

Table 2. Recovery of staphylococcal enterotoxin A added at 1 ng/g to some foods

Food	Vol. ext assayed, μL	SEA, ng/mL Detected[a]	SEA, ng/mL Av.	SEA recd, %
Cheese	100	0.80	0.75	72
	200	0.70		
Pasta	100	0.50	0.52	63
	200	0.53		
Sardine	100	0.90	0.95	97
	200	1.00		
Salmon	100	0.90	0.80	105
	200	0.70		
Sausage	100	0.70	0.63	75
	200	0.55		
Salami	100	0.50	0.45	52
	200	0.40		

[a] Average of duplicate determinations.

dence that the radioimmunoassay is measuring SEA in the sample. Non-specific inhibition due to the food itself was negligible in most cases; the inhibitory effect of extracts of pasta, cheese, sardine, salmon, egg noodles, and fortune cookies not treated with enterotoxin ranged between 0 and 5% reduction of ^{125}I-labeled toxin uptake. A desirable test for SEA detection should require simple sample preparation and an ability to detect 100–200 ng SEA/100 g food (15). Samples of food to which 100 ng enterotoxin A had been added gave positive results in the test (Table 2). Average recovery of the toxin ranged from 52 to 105%, and there was good agreement with analytical volumes of 100 and 200 μL. Because approximately 94% SEA was recovered when added to food extracts, the low recovery of toxin added directly to these foods, particularly salami and pasta, must be attributed to losses during the extraction procedure. We have been able to improve the recovery of SEA from these foods by re-extracting the residue twice and combining the extracts.

REFERENCES

(1) Casman, E. P., & Bennett, R. W. (1965) *Appl. Microbiol.* **13,** 181–189
(2) Bergdoll, M. S., & Reiser, R. (1980) *J. Food Protection* **43,** 68–72
(3) Dickie, N., Park, C. E., & Robern, H. (1976) *J. Clin. Pathol.* **29,** 833–835
(4) Pober, Z., & Silverman, G. J. (1977) *Appl. Environ. Microbiol.* **33,** 620–625
(5) Freedlender, A. E., Fyhrquist, F., & Hollemans, H. J. G. (1974) in *Methods of Hormone Radioimmunoassay*, B. M. Jaffee & H. R. Behrman (Eds), Academic Press, Inc., New York, NY, pp. 455–469
(6) Miller, B. A., Reiser, R. F., & Bergdoll, M. S. (1975) *Appl. Environ. Microbiol.* **36,** 421–426
(7) Robern, H., Gleeson, T. M., & Szabo, R. A. (1978) *Can. J. Microbiol.* **24,** 436–439
(8) Catt, K., & Tregear, G. W. (1967) *Science* **158,** 1570–1572
(9) Robern, H., Stavric, S., & Dickie, N. (1975) *Biochim. Biophys. Acta* **393,** 159–164
(10) Rodbard, D., Lennox, R. H., Wray, H. L., & Ramseth, D. (1976) *Clin. Chem.* **72,** 350–358
(11) Rodbard, D. (1978) *Radioimmunoassay and Related Procedures in Medicine*, Vol. 2, International Atomic Energy Agency, Vienna, Austria, pp. 21–36
(12) Rodbard, D. (1978) *Anal. Biochem.* **90,** 1–12
(13) Robern, H., Stavric, I., & Dickie, N. (1975) *Biochim. Biophys. Acta* **393,** 148–158
(14) Rodbard, D., Munson, P. J., & De Lean, A. (1978) *Radioimmunoassay and Related Procedures in Medicine*, Vol. 1, International Atomic Energy Agency, Vienna, Austria, pp. 469–503
(15) Reiser, R., Conaway, D., & Bergdoll, M. S. (1974) *Appl. Microbiol.* **27,** 83–85

Manual Headspace Gas-Solid Chromatographic Determination of Sub-Parts per Trillion Levels of Acrylonitrile in 3% Acetic Acid

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A procedure was developed for determining acrylonitrile (AN) in 3% acetic acid, the solvent used to simulate carbonated beverages. Manual headspace sampling, a detector output amplifier/filter, and vapor phase enhancement using an ionic material were combined to give a detection limit in the low parts per trillion range. AN standards were analyzed using the method, and the results are presented.

Headspace gas chromatography (GC) is well suited for the determination of volatile components in foods and food-simulating solvents. Novel approaches to headspace sampling for trace volatile compounds have been reported, with major emphasis on the determination of vinyl chloride (VC) in many matrices. The sampling of headspace gases from plastics sealed in "hot jars" has become an official ASTM procedure for determining volatiles in flexible barrier films (1). Rosen et al. (2) described a procedure using the hot jar technique to liberate residual VC from the polymer with subsequent analysis of the headspace for VC using GC/mass spectrometry. Dennison et al. (3) described a sparging technique in which VC was removed from the polymer matrix, trapped and concentrated in a holding solvent, and analyzed by GC

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using a manual headspace sampling technique. Depending on the toxicity of the volatile component, it is often desirable to measure small quantities with high precision. The analyst wants a rapid, simple procedure. Selective detector systems are presently available for use in reaching these goals. Our work (4), as well as that of others (5, 6), has followed this course in developing methods to determine acrylonitrile (AN).

The lowest reliable limit reported for the determination of AN in 3% acetic acid, a solvent used to simulate carbonated beverages, was 10 ppb (7, 8). Using headspace methodology, the detection limit was decreased to 1 ppb (M. E. Brown, 1978, unpublished data, Food and Drug Administration, Washington, DC 20204). Now a procedure has been developed, using a nitrogen selective detector and a manual headspace GC technique, for determining part per trillion quantities of AN in 3% acetic acid. This sensitive procedure incorporates a detector signal amplifier/filter and the use of an ionic compound to enhance the AN concentration in the headspace.

Experimental

Apparatus

(a) *Headspace sample vials.*—23 mL vials with 12 × 20 mm finished tops and aluminum seals (Shamrock Glass Co., 201 E 10th St, Marcus Hook, PA 19061), or equivalent. Tuf-Bond Teflon/silicon disks, No. 12720 (Pierce Chemical Co., Rockford, IL 61105), or equivalent.

(b) *Syringes.*—100 μL Hamilton 700 series, or equivalent. Two and 5 mL Pressure-Lok, series A-2 gas syringes (Precision Sampling Corp., Baton Rouge, LA 70815), or equivalent.

(c) *Forced air oven.*—Stable-Therm constant temperature cabinet (Blue M Electric, Blue Island, IL 60406), or equivalent.

(d) *Gas chromatograph.*—Perkin-Elmer Model 3920 with nitrogen-phosphorus (N-P) selective detector (Perkin-Elmer Corp., Norwalk, CT 06856), or equivalent. Detector parmeters: jet potential 3 (N-P mode), bead rheostat setting (400–700) as necessary to give 10% full scale deflection for AN from 2 μL injection of 0.05 μg/mL AN in 3% acetic acid solution (100 pg AN). Electrometer setting 4 × 1 with 5 mV signal output (4×10^{-12} A full scale). Operating conditions: temperatures (°C)—injection port 175; oven, column A 115, column B 125; interface 215. Gas flow (mL/min)—helium carrier, column A 25, column B 20; hydrogen 5; air 125.

(e) *Chromatographic columns.*—Column A, coiled 6 ft × 2 mm id glass, packed with 80–100 mesh Chromosorb 101; column B, coiled 3 ft × 2 mm id glass packed with 100–120 mesh Chromosorb 108.

(f) *Spectrum filter and amplifier.*—1021A (Spectrum Scientific Corp., Newark, DE 19711), or equivalent.

Reagents

(a) *Acetic acid.*—3%. Baker Analyzed Reagent (J.T. Baker Chemical Co., Phillipsburg, NJ 08865), or equivalent. Prepare fresh weekly.

(b) *Acrylonitrile (AN).*—99% pure (Polysciences, Inc., Warrington, PA 18976), or equivalent. Caution: AN is a teratogen and carcinogen. Use safety precautions.

(c) *Standard solutions.—Stock solutions.*—About 2000 ppm AN in 3% acetic acid. In hood, transfer 20 mL 3% acetic acid into tared headspace vial, cap, and weigh to nearest 0.1 mg. Transfer 50 μL AN into vial using 100 μL syringe; cap vial with fitted butyl septum, reweigh, and seal vial. Calculate AN concentration in stock solution.

Intermediate solution.—About 10 ppm. Add 0.1 mL stock solution to 20 mL 3% acetic acid.

Working solution.—About 0.05 ppm. Add 0.1 mL intermediate solution to 20 mL 3% acetic acid. Seal vial with Teflon-faced septum. Working solution is stable for 2 weeks if kept sealed at room temperature.

Preparation of Standards and Samples

Headspace standards.—Transfer 4.5 g anhydrous Na_2SO_4 to headspace vial. Pipet 15 mL 3% acetic acid into vial. Add appropriate volume (μL) intermediate or working solution to vial, using 100 μL syringe. Quickly seal vial with Teflon-faced septum. Transfer vial to 90°C forced air oven. Periodically shake vial until salt dissolves (ca 1.5 h).

Headspace samples.—Add 15 mL sample to headspace vial containing 4.5 g anhydrous Na_2SO_4. Seal vial with Teflon-faced septum, and prepare for analysis as described for headspace standards.

Analysis of Headspace Solutions

The headspace analysis of samples and standards is performed in the same manner. After complete dissolution of salt, allow 0.5 h for complete phase equilibrium to occur. With previously heated syringe (in same oven), pierce vial septum, draw up full volume of syringe once, and then draw desired headspace volume. Let needle remain in vial headspace for at least

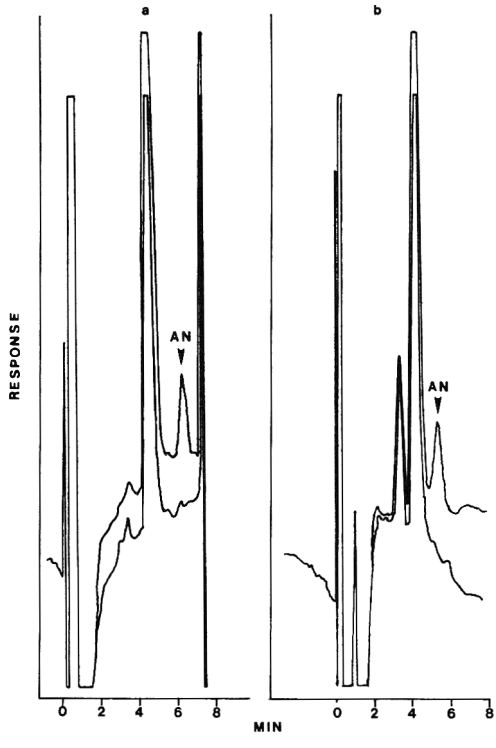

Figure 1. Chromatograms of headspace sampled from 15 mL 100 ppt AN in 3% acetic acid with 4.5 g Na_2SO_4 and their corresponding blanks. a, 5 mL headspace analyzed using Chromosorb 101; b, 5 mL headspace analyzed using Chromosorb 108. Detector attenuation 2 × 1. Spectrum filter cut-off frequency 0.02 Hz. Lower chromatogram is blank.

1 min in closed oven. Quickly close syringe valve, remove syringe, and inject headspace gases into gas chromatograph. Under operating conditions described, AN elutes in ca 6 min on columns A and B.

AN in samples is quantitated by external standardization. The linearity of this system has been demonstrated from 0.05 to 0.6 ppb using headspace standards.

Results and Discussion

The manual headspace technique allows large volumes of sample headspace to be introduced onto the GC column. Water vapor, volatile impurities, and acetic acid are the major components of the headspace gases injected. The injection size and the highly polar nature of water and acetic acid require a hydrophobic column packing material of high capacity. Chromosorb 101 and 108 have been used to separate AN from the 3% acetic acid matrix using a direct liquid injection technique (8, 9). Chromosorb 101 resolves AN from early eluting liquids, such as water, while Chromosorb 108 resolves AN from late eluting liquids, i.e., acetic acid. The use of both columns has been incorporated in this work. Figure 1 shows chromatograms of headspace from 15 mL 0.1 ppb AN in 3% acetic acid with 4.5 g Na_2SO_4.

The initial developmental work at the part per trillion level showed an inadequate signal-to-noise ratio for AN when 5 mL headspace aliquots were injected. The value of enhancing the AN headspace concentration using a compound of high ionic strength to shift the AN partition equilibrium toward the headspace was therefore investigated. Sodium chloride concentrations of 1.5, 3, and 4.5 g/15 mL 0.2 ppb AN in 3% acetic acid were evaluated by headspace GC. As expected, the higher the salt content, the higher the AN headspace concentration. Addition of 4.5 g NaCl to 15 mL 0.2 ppb AN in 3% acetic acid approximately doubled the AN headspace concentration. A further study with 4.5 g Na_2SO_4 added to 15 mL 0.2 ppb AN in 3% acetic acid tripled the AN concentration in the headspace. The addition of sodium sulfate enhanced AN headspace concentration so that the desired response for AN above baseline noise was attained. Figure 2 compares the increase in response seen for AN when equal headspace aliquots are sampled and analyzed by GC. The 13 mm AN response observed with no salt increases to 38 mm with the addition of 4.5 g Na_2SO_4.

Figure 2 also shows the level of high frequency noise associated with the high sensitivity inherent to the N-P detector. This noise is greatly reduced by using a noise filter between the electrometer and recorder. Using the filter, high frequency noise was dampened sufficiently at cutoff frequencies of 0.05 and 0.02 Hz. Figure 1 shows chromatograms utilizing the 1021A filtering capability.

To avoid the chromatographic interferences derived from grey butyl septa, only Teflon-faced disks were used for headspace sampling. The responses of headspace solutions analyzed using the Teflon-faced disks were stable. As many as 3 samplings of the same vial headspace gave the same response for AN even when the solution was maintained at 90°C up to 5 h. Although sealed AN solutions were apparently stable, they were analyzed on the day of their preparation and then discarded.

After each injection, the syringe and needle were flushed with acetone to remove any septum particles clogging the needle. Care was taken

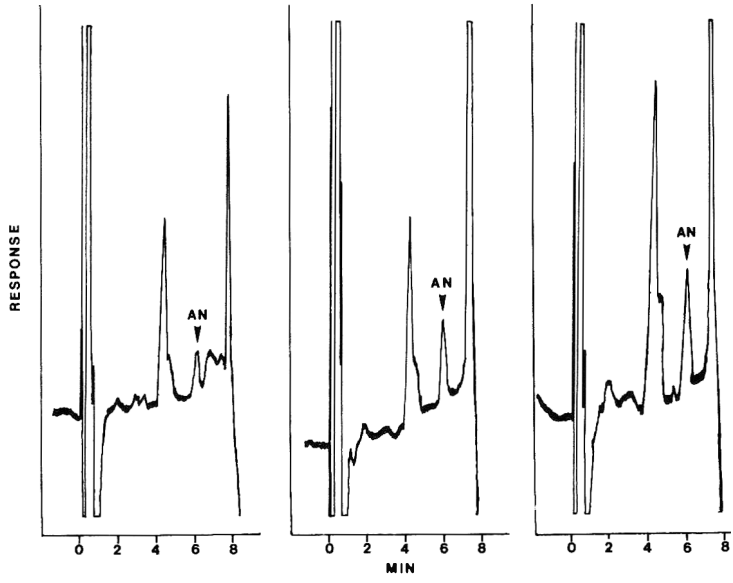

Figure 2. Chromatograms of 5 mL headspace sampled from 15 mL 200 ppt AN in 3% acetic acid with (left to right) no added salt, 4.5 g NaCl, and 4.5 g Na_2SO_4. Detector attenuation 4 × 1; no signal filtering was used.

with the injection technique to prevent disfiguration of the needle tip. A burr on the needle tip quickly shredded the septum and clogged the gas-tight syringe needle.

Rapid analysis of a series of headspace samples was hindered by a late-eluting compound. This was overcome by bracketing injections before and after the interference, and was most easily accomplished using the Chromosorb 108 column.

All results reported represent analyses of standard parts per trillion range AN in 3% acetic acid solutions. Our laboratory only had access to samples in the parts per billion range, and these samples were not analyzed using this procedure since their concentrations could be easily determined using other, less sensitive procedures.

Detector linearity, expressed as mm response for AN injected vs AN concentration in solution, was demonstrated from 50 to 600 ppt. Linear correlation coefficients of 0.993 and 0.999 were observed using the Chromosorb 108 and 101 columns, respectively. The response observed for 5 mL headspace injected from duplicate samplings of three 100 ppt solutions of AN in 3% acetic acid was 28.5 mm (SD 1.41) for the Chromosorb 108 column and 33 mm (SD 2.04) for the Chromosorb 101 column. Precision and linearity were reinforced further by using 2 different injection volumes, 2 and 5 mL.

In summary, a procedure is presented which permits the determination of parts per trillion levels of AN in 3% acetic acid. The procedure is relatively simple and gives precise and accurate results for standard solutions.

REFERENCES

(1) *Annual Book of ASTM Standards* (1979) American Society for Testing and Materials, Philadelphia, PA, F151-72, Part 21, pp. 372–377
(2) Rosen, J. D., Morano, J. R., Pareles, S. R., Giacin, J. R., & Gilbert, S. G. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 700–702
(3) Dennison, J. L., Breder, C. V., McNeal, T. P., Snyder, R. S., Roach, J. A., & Sphon, J. A. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 813–819
(4) McNeal, T. P., & Breder, C. V. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 270–275
(5) DiPasquale, G., Dilorio, G., & Capaccioli, T. (1978) *J. Chromatogr.* **160,** 133–140
(6) Gawell, G. B.-M. (1979) *Analyst* **104,** 106–110
(7) *Fed. Regist.* (Sept. 23, 1977) **42,** 48528, 48544
(8) McNeal, T. P., Brumley, W. C., Breder, C. V., & Sphon, J. A. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 41–46
(9) Brown, M. E., Breder, C. V., & McNeal, T. P. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1383–1388

FOR YOUR INFORMATION

James P. Minyard is AOAC President for 1982

James P. Minyard, Jr, head of the Mississippi State Chemical Laboratory and Professor of Chemistry at Mississippi State University, is President of AOAC for 1982. Dr. Minyard is active in research and numerous professional societies, and is listed in *Who's Who in the South, Leaders in American Science,* and *American Men of Science.*

Dr. Minyard's research is primarily concerned with pesticide residues, pesticide degradation, and biological control of the cotton boll weevil. He and colleagues at the Boll Weevil Research Laboratory at Mississippi State isolated and identified the sex attractant compounds by which the male boll weevil attracts its mate. He has published over 35 scientific papers in national and international journals, is co-inventor on two patents, and is coauthor of a chapter in the book, *Chemicals Controlling Insect Behavior.*

It is often said that if you want a job done, ask a busy man or woman to do it. James P. Minyard is a classic case in point. A member of AOAC since 1960 and an energetic participant since his appointment as Associate Referee on DDT in 1968, Dr. Minyard has served on the Interagency Committee, the Committee on Classification of Methods, the Committee for Collaborative Studies, and the Committee on International Cooperation, as Chairman of the Editorial Board, and as a member of the Board of Directors.

In addition to AOAC, he is active in many other professional societies including the Pesticide Chemistry Division of the American Chemical Society (Chairman 1977, and Fellow 1974), the American Association for the Advancement of Science, the Mississippi Academy of Sciences, Sigma Xi, the Newcomen Society in North America, the American Oil Chemists' Society, American Feed Control Officials (President 1975), the Association of American Plant Food Officials, and Phi Lambda Upsilon.

Dr. Minyard is an ex-officio member of the Mississippi Agricultural and Industrial Board and a member of the Starkville Chamber of Commerce. A native Mississippian, he received his B.S. in chemistry from Mississippi State University in 1951, studied at the California Institute of Technology, and received a Ph.D. in organic chemistry from Mississippi State University in 1967. Before that, he had been employed by the Mississippi State Chemical Laboratory and the University as an instructor and chemist, and by the U.S. Department of Agriculture as a research chemist.

Dr. Minyard is married to the former Mary Louise Whitesell and has five children. He is an active member of the Starkville First United Methodist Church, the Starkville Chapter of the Full Gospel Business Men's Fellowship International, and does lay work in the Methodist Church throughout Mississippi.

AOAC Welcomes Its Newest Private Sustaining Members

AOAC welcomes these latest additions to the growing list of companies aware of the need to support an independent methods validations association: Agrico Chemical Co., Baltimore, MD; Bacardi Corp., San Juan, PR; Coca-Cola Co., Atlanta, GA; Corn Products (CPC International), Summit-Argo, IL; Duphar BV, The Netherlands; Endo Laboratories, Inc., Garden City, NY; FBC Ltd, UK; FMC Corp., Princeton, NJ; E & J Gallo Winery, Modesto, CA; O. M. Scotts & Sons, Inc., Marysville, OH; and Technicon Industrial Systems, Tarrytown, NY.

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AOAC to Hold 7th Annual AOAC Spring Training Workshop

AOAC will holds its 7th Annual Spring Workshop and Exposition April 13–15, 1982, at the Fairmont Hotel, New Orleans, LA. There will be 14 main sessions, 4 supplementary symposia and seminars, a wine and cheese party, and an exhibition. Co-chairpersons are: Nicole Hardin, Food and Drug Administration, 4298 Elysian Fields Ave, New Orleans, LA 70122, 504/589-2471, and Hershel Morris, Louisiana Dept of Agriculture, PO Box 16390-A, University Station, Baton Rouge, LA 70893, 504/388-2755.

Session topics and chairmen will be: *Animal Feeds* (including drugs and antibiotics), chaired by Alan Hanks, Agricultural Analytical Services Lab., Texas A & M University, College Station, TX 77840, 713/845-4111. This session will include a seminar on monensin. For additional information, contact Alan Hanks, or William Morris, FDA, 721 19th St, U.S. Customs House, Room 500, Denver, CO 80202, 303-837/4223. *Fertilizers*, chaired by C. H. McBride, USS Agricultural Chemicals, 685 DeKalb Industrial Way, Decator, GA 30033, 404/292-2525. *Pesticide Formulations*, chaired by Edwin Jackson, Mississippi State Chemical Lab., PO Box CR, Mississippi State, MS 39762, 601/325-3324. *Pesticide Residues*, chaired by W. E. McCasland, Texas Dept of Agriculture, PO Box 1119, Benham, TX 77833, 713/836-5641. In conjunction with the pesticide residues and pesticide formulations sessions, the Chemical and Biological Investigation Branch of the EPA Laboratory at Beltsville, MD, is sponsoring discussions on sampling techniques and methodology; for additional information, contact Adrian Burns, 301/344-2015, or Paul Jung 301/344-2246, EPA/CBIB, Beltsville, MD 20705. *Analysis for Toxicological Research*, chaired by Malcolm C. Bowman, National Center for Toxicological Research, FDA, Jefferson, AR 72079, 501/541-4000. *Analysis Related to Seafood Quality*, chaired by Beverly Smith, National Marine Fisheries Laboratory, PO Box 1207, Pascagoula, MS 39567, 601/762-4591. *Mycotoxins*, chaired by Louise Lee, USDA/SRRC, PO Box 19687, New Orleans, LA 70179. *Environmental Monitoring*, chaired by James P. Wood, Carbon Systems, Inc., 1287 Main St, Baton Rouge, LA 70802, 504/343-3353. *Laboratory Automation*, chaired by Robert Beine, Division of Regulatory Services, University of Kentucky, Lexington, KY 40506, 606/257-1656. *Laboratory Quality Assurance*, chaired by Patricia Smith, Woodson-Tenent Laboratories, PO Box 2135, Memphis, TN 38101, 901/525-6333. *Veterinary Toxicology*, chaired by Steven S. Nicholson, 231 Knapp Hall, Louisiana State University, Baton Rouge, LA 70803, 504/388-4141. *Drug and Antibiotic Residues in Animal Tissue*, chaired by Raymond Ashworth, U.S. Dept of Agriculture, Food Safety and Inspection Service, Beltsville, MD 20705. *Environmental Contamination in Food Such as Meat Products* (2 sessions), chaired by Raymond Ashworth and Robert Epstein (address same as above).

Additional symposia and seminars to be held in conjunction with the AOAC Spring Workshop are as follows: *Food Toxicology*, sponsored by the Association of Food and Drug Officials; for additional information, contact Martha Rhodes, Florida Dept of Agriculture, Mayo Bldg, Tallahassee, FL 32304, 904/488-0670, or John Turner, Food and Drug Administration, 900 Madison Ave, Baltimore, MD 21201, 301/962-3790. *Pesticide Enforcement Laboratory Procedures* (*Residue and Formulation*), a training program for analysts involved in the pesticide enforcement grant program, sponsored by EPA National Enforcement Investigations Center; for additional information, contact Dean Hill, EPA/NEIC, Box 25227, Denver, CO 80225, 303/234-3751.

For display booth space at this meeting, contact Joseph Ford, U.S. Dept of Agriculture, Pesticide Monitoring Laboratory, PO Box 989, Gulfport, MS 39501.

Reminder of Deadlines for Nominations for Awards

The following deadlines have been set for nominations for awards: 1982 Fellow of the AOAC Award, March 1, 1982; 1982 Harvey W. Wiley Award, April 1, 1982; and 1982–1984 Scholarship Award, May 1, 1982.

The Fellow of the AOAC award was established in 1961 to recognize those persons giving meritorious service to the Association. Eligible candidates have performed notably for 10 years or more, usually as Officers, Referees, or Committee members. Fellows are selected by the Committee on Fellows and approved by the Board of Directors. Selection is made from a list which includes eligible candidates, their contributions, and the point count assigned to each candidate according to

an established point system. The Committee is also informed of any nominations received.

Free Guide Available for Claims Substantiation for Disinfectants and Sanitizers

Hill Top Research, Inc. of Cincinnati, OH, announces the availability, upon request, of a booklet, *Disinfectants and Sanitizers: Guide to Claims Substantiation Testing*. The *Guide* outlines testing required or recommended by EPA in support of antimicrobial efficacy claims for various types of germicidal products. To obtain one, contact Ward L. Billhimer, Director of Operations, Microbiological Services Division, Hill Top Research, Inc., PO Box 42501, Cincinnati, OH 45242, or phone 512/831-3114.

Meetings

April 13–15, 1982: 7th Annual AOAC Spring Training Workshop and Exposition, Fairmont Hotel, New Orleans, LA. (For details, see article above.)

October 25–28, 1982: 96th Annual AOAC Meeting, Shoreham Hotel, Washington, DC. For additional information, contact Kathleen Fominaya, AOAC, 1111 N 19th St, Arlington, VA 22209; telephone 703/522-3032.

July 17–23, 1983: SAC 83—An International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, Edinburgh, Scotland. Organized by the Analytical Division of the Royal Society of Chemistry. To include: one-day UPDATE courses, an exhibition, a social program and tours, lectures, contributed papers, and poster sessions. To be covered—techniques: atomic spectroscopy, biochemical methods, chromatography, electroanalysis, electrophoresis, enzyme techniques, immunoassay, mass spectrometry, microanalysis, molecular spectroscopy, photoacoustic spectrometry, probe methods, radiochemistry, sample preparation (preconcentration and separation), thermal methods, and X-ray methods; materials: agricultural, biological, clinical, environmental, food and drink, geological, pharmaceutical, surfaces, metallurgical, and water and effluents; other aspects: automation, data processing, process control, microcomputers, microprocessors, quality control, historical, and industrial. For additional information, contact P. E. Hutchinson, Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London, W1V OBN, UK.

July 27–30, 1983: 3rd International Conference on the Instrumental Analysis of Foods and Beverages—Recent Developments, the Corfu Hilton, Corfu, Greece. Under the auspices of the Greek Ministry of Agriculture. Co-sponsored by the Agriculture and Food Chemistry Division of the American Chemical Society, the Institute of Food Technologists, and the Society of Flavor Chemists, Inc., in association with the Department of Food Science of the University of Ioannina, Greece, and the Cereal Institute of Thessaloniki, Greece. For information, contact C. J. Mussinan, IFF R & D, 1515 Highway 36, Union Beach, NJ 07735; telephone 201/264-4500.

BOOK REVIEWS

Methods in Vitamin B_6 Nutrition – Analysis and Status Assessment. Edited by J. E. Leklem and R. D. Reynolds. Published by Plenum Press, New York and London, 1981. xi + 401 pp. Price: $49.50.

This book was written for the analytical chemist, biochemist, clinical chemist, food technologist, and nutritionist. The topic is very well covered and the bibliographies, old and current, are complete and make this an excellent reference book.

The volume is divided into three sections. The first section covers historical background, chemical properties, and structures of vitamin B_6 and its different forms, and describes the chemical, microbiological, and most recent instrumental methodologies used to determine vitamin B_6 and its isomers or metabolites. The second section deals with application to different matrices and serves as a powerful tool for the analyst involved in methods development work or the scientist involved in measurements of vitamin B_6 and its isomers or metabolites. Each method assessment is weighed to provide a helpful guide for the scientist in the field. The final section deals with recommended methods for vitamin B_6 determination, evaluation of established methods, and recommendations for future methodological development.

A research chemist can read this book, gain helpful hints, and perform the assay, as well as pursue a direction in developing a method of analysis.

In summary, this informative and well written book is highly recommended as a reference book.

MARTIN BUENO

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Liquid Chromatographic Analysis of Food and Beverages. Volume 1. Edited by George Charalambous. Published by Academic Press, Inc., New York, NY 10003 and Academic Press, Inc. (London) Ltd, London, UK, 1979. 236 pp. Price: $16.00.

Papers presented April 1-6, 1979, in Honolulu, Hawaii, at a symposium on the analysis of foods and beverages by HPLC comprise this book. The symposium was organized by the Flavor Subdivision of the American Chemical Society at its 177th National Meeting in conjunction with the Chemical Society of Japan, with official participation of the Royal Australian Chemical Institute, The New Zealand Institute of Chemistry, and the Chemical Institute of Canada.

The book presents a good assemblage of actual HPLC analysis and methodology for a variety of food components: citrus products, amino acids, aflatoxins, anthocyanins, capsaicins, vitamins, pesticides, essential oils, Amadori compounds, and germinated soybeans and flours. It contains up-to-date information on the use of microprocessor-based variable wavelength detectors, dual detection systems, fluorescent detectors, and improved data collection. The scientist involved with analysis of foods would find this book useful because HPLC analysis very often offers superior accuracy, precision, and identification.

Volume 2, in the process of publication, contains other presented papers not available for Volume 1. Six chapters in Volume 2 cover theory and application of reverse phase HPLC.

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NEW PUBLICATIONS

The Quality of Foods and Beverages, Chemistry and Technology, Volumes 1 and 2. Edited by George Charalambous and George Inglett. Published by Academic Press, Inc., 111 Fifth Ave, New York, NY 10003, 1981. Vol. 1: 456 pp., $29.50, ISBN 0-12-169101-2. Vol. 2: 400 pp., $26.50, ISBN 0-12-169102-0.

These two volumes present the proceedings of a conference on the quality of foods and beverages held in Athens, Greece, in 1981, under the auspices of the Greek Ministry of Agriculture. Fifty-eight authors discuss the recent findings of 105 scientists from 20 countries on the chemistry and technology underlying the quality of foods and beverages. Effects on quality of taste and flavor interactions with food components, as well as those of complex carbohydrates, cyclodextrins, hydrolyzed proteins, and plant additive water-lipid interactions; new sweeteners and the search for sweetness criteria; flavor contribution of volatile sulfur compounds, carotenoids, and cell-mediated immune responses; new mechanized plating instrument for viable microorganism counts; novel proteins; advances in shoyu research, legume processing, cheese flavor research and breadmaking; and developments in analytical methodology in the fields of scotch whisky flavor, wines, vinegar, alcoholic beverages, and coffee.

1982 USAN/USP Dictionary of Drug Names. Published by U.S. Pharmacopeial Convention, Inc.; distributed by USAN Division, USP Convention, Inc, 12601 Twinbrook Pkwy, Rockville, MD 20852, 1981. 615 pp. Price: $25.00 in US (for 1–10 copies), $21.75 in US (for 11–24 copies).

Since 1961, the United States Adopted Names (USAN) system has been operating to assign a single, nationally recognized name for every new substance that shows promise as a drug. The *U.S. Pharmacopeia*, the *National Formulary*, and the Food and Drug Administration use USAN as established or official drug names. Information included among the 17 000 entries is USAN, pronunciation guides, compendial and other generic names, brand names, therapeutic categories, manufacturers, investigational code designations, CAS registry numbers, International Nonproprietary Names (INN), and molecular weights. To facilitate literature search, the multiple CAS registry numbers are annotated to relate them to various forms of a compound. Of the 2881 INN included, 2462 are independent entries because the compounds concerned are covered by no other entries in the book.

Handbook of Toxic Fungal Metabolites. By Richard J. Cole and Richard H. Cox. Published by Academic Press, Inc., 111 Fifth Ave, New York, NY 10003, 1981. 924 pp. Price: $79.00. ISBN 0-12-179760-0.

Facilitating the identification of known or related mycotoxins is the purpose of this handbook. It provides a comprehensive accumulation of chemical, physical, spectral, and biological data on toxic fungal metabolites and related chemicals that are scattered throughout the literature. The book will also be particularly useful to scientists interested in some aspect other than the toxic nature of a particular chemical species or related species. Where possible, actual copies of UV, IR, ^{1}H-NMR, ^{13}C-NMR, and mass spectra are presented to facilitate spectral identification of known mycotoxins or related metabolites. The handbook is primarily oriented toward fungal metabolites that elicit a toxic response in vertebrate animals; however, metabolites that show little or no known acute toxicity are included because of their chemical or close biosynthetic relationship to a toxin or group of toxins.

Quality Control in Analytical Chemistry. By Gerrit Kateman and Frans W. Pijpers. Published by Wiley Professional Books-By-Mail, John Wiley & Sons, Inc., Dept 0199, Somerset, NJ 08873, 1981. 320 pp. Price: $40.00. ISBN 1-46020-6.

Techniques for improving the quality of analytical methods, sampling, and data handling are described in this guide. Questions such as which quality parameters can be improved and which one should be improved first are answered on the basis of statistical techniques. In addition, effective ways to control and select analytical procedures and organize analytical work for maximum benefit are discussed.

Aquatic Chemistry, 2nd Edition. An Introduction Emphasizing Chemical Equilibria in Natural Waters. By Werner Stumm and James Morgan. Published by Wiley Professional Books-By-Mail, John Wiley & Sons, Inc., Dept 0199, Somerset, NJ 08873, 1981. 780 pp. Price: $45.00. ISBN 1-04831-3.

The latest edition of this text responds to aquatic chemistry's maturation in unifying concepts, applications, and techniques, and to its burgeoning data. Chemical equilibrium is retained as the basic framework essential for an understanding of aquatic chemistry with increased attention given to steady-state and dynamic models employing mass-balance approaches and kinetic information. Additional sections are devoted to organic compounds in natural water systems and to the use of stable and radioactive isotopes in characterizing physical and chemical processes. Significant advances in marine chemistry, solid-solution interface, and kinetic considerations are also covered.

Digital Computers in Analytical Chemistry, Parts I & II. Edited by J. B. Justice, Jr, and T. L. Isenhour. Published by Academic Press, Inc., 111 Fifth Ave, New York, NY 10003, 1981. Part I: 384 pp, $56.00. ISBN 0-12-786786-4; Part II: 416 pp, $56.00, ISBN 0-12-786787-2. Price for Parts I & II purchased together, $100.00; not valid in Australia or New Zealand.

The evolution of computer use in analytical chemistry is traced in this two-volume work. Part I surveys 1950–1969 and covers the development of large-scale data processing facilities in the United States. It emphasizes work on off-line numerical processing of data. Part II covers 1970–1978 and discusses the evolution of minicomputers and the growth of data acquisition.

Organic Trace Analysis by Liquid Chromatography. By James F. Lawrence. Published by Academic Press, Inc., 111 Fifth Ave, New York, NY 10003, 1981. 284 pp. Price: $34.00. ISBN 0-12-439150-8.

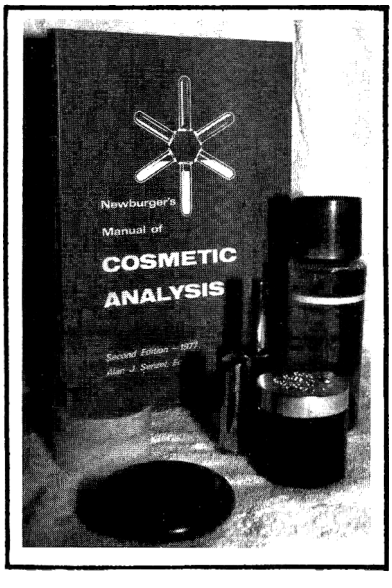
Analysts involved in the determination of trace organics in many different substrates will find this book to be of value. It will be of particular interest as a teaching aid for those entering the field of trace analysis with the intention of using liquid chromatography. Chapter titles are: General Considerations in Developing a Trace Analytical Technique Employing Liquid Chromatography, Pumping Systems, Sampling Technique and Injection Ports, Chromatography Columns and Packing Materials, Detectors, Chromatography, Chemical Derivatization, Sample Extraction Cleanup, Approach to Methods Development, and Routine Analysis, and Applications.

Carotenoids as Colorants and Vitamin A Precursors, Technological and Nutritional Applications. Edited by J. Christopher Bauernfeind. Published by Academic Press, 111 Fifth Ave, New York, NY 10003, 1981. 924 pp. Price $95.00. ISBN 0-12-082850-2.

The existing knowledge on the application of carotenoids to food and to the feed of animals, fish, poultry, and birds is assembled in this book. The use of carotenoids in medicine, in the coloring of pharmaceutical and cosmetic products, their unique role as photoconductors, and analytical methodology are discussed. The volume illustrates the imperfect state of knowledge on the qualitative and quantitative distribution of carotenoids in natural foods and calls for an effort in applying modern analytical methodology to carotenoid determination.

Newburger's Manual of
Cosmetic Analysis

- Chapters on analytical techniques: gas chromatography, other chromatographic techniques, and spectroscopy
- Chapters on analysis of creams and lotions, lipsticks, nail lacquers and extenders, hair fixatives and dyes, cold wave solutions and neutralizers, antiperspirants and deodorants, sunscreens
- Chapters on cosmetic microbiology, colors, perfumes and fragrances, preservatives, and toothpastes



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