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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

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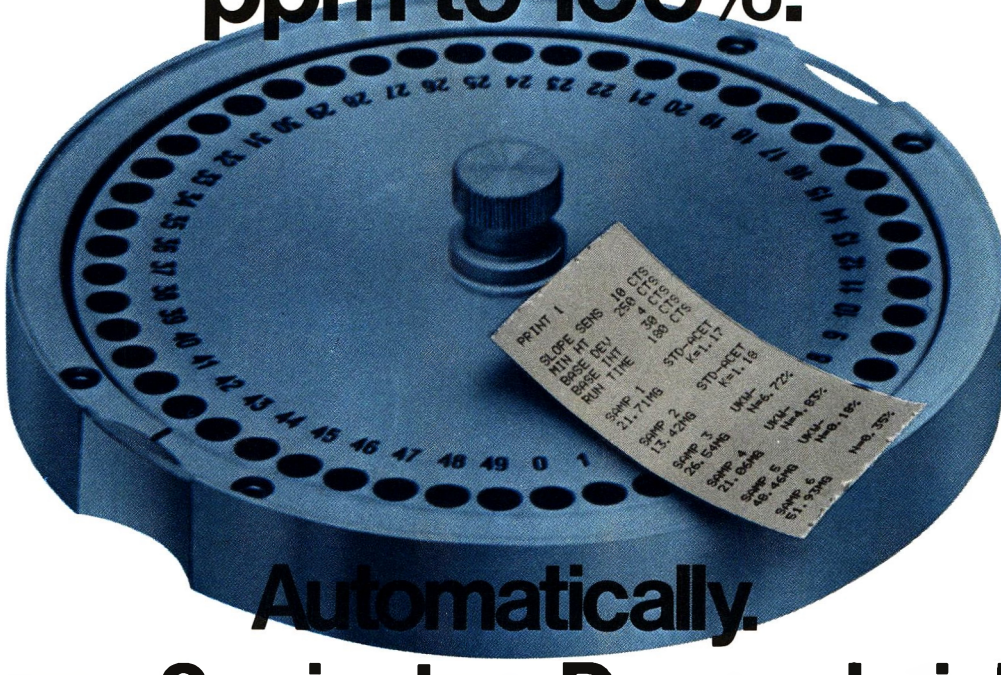

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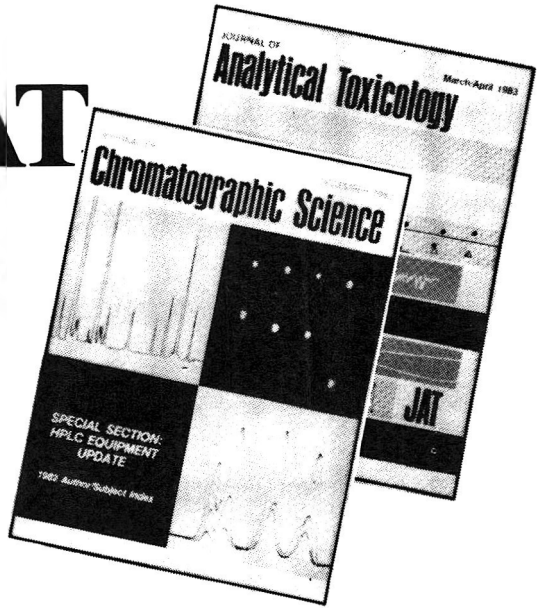

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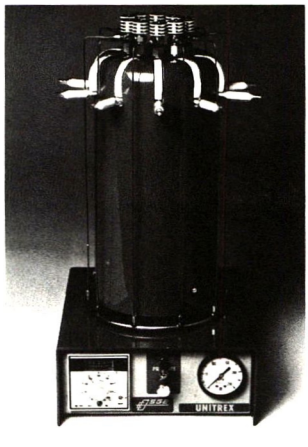

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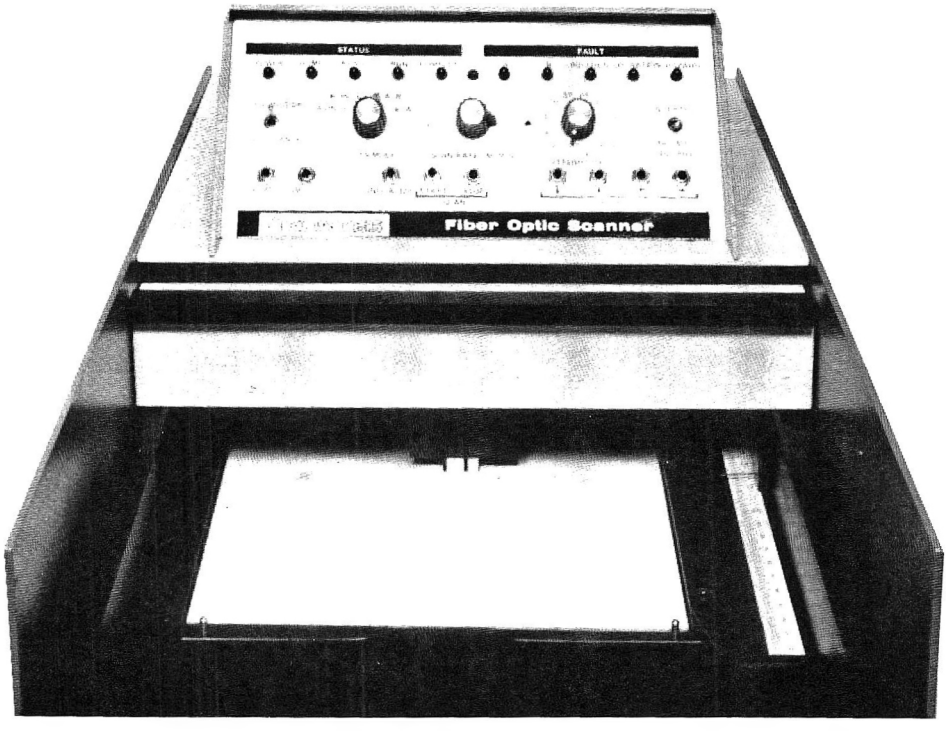

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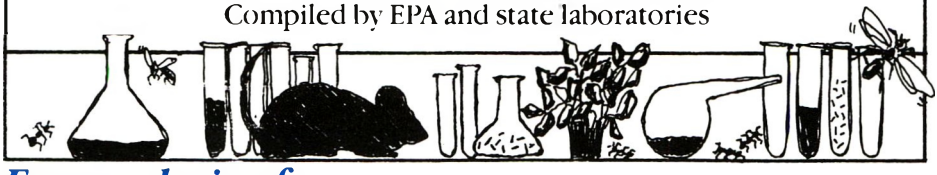

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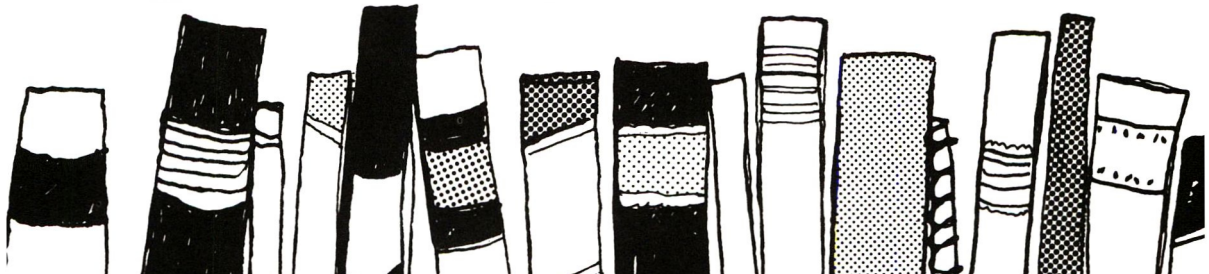

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JOURNAL of the ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

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Vol. 66 NOVEMBER 1983 NO. 6

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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

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PESTICIDE RESIDUES

Novel Method for Estimation of Chlorinated Pesticide Residues in Milk

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A new, simple, and rapid procedure for determining chlorinated pesticide residues in milk is described. The entire acetonitrile extract from milk is passed through a 0.5 g activated charcoal chromatographic column. Chlorinated pesticides adsorbed on the charcoal are eluted with 100 mL acetone-hexane (1 + 1). The eluate is washed with water and 1% sodium carbonate solution, and chlorinated pesticides are extracted with hexane. The extract is concentrated and measured by electron capture gas chromatography. Recoveries from 50 mL milk samples fortified with 0.04-1.6 μg of different BHC isomers, 0.05-2 μg DDT and its metabolites, and 0.05-0.5 μg dieldrin ranged from 86.9 to 103.2%.

Activated charcoal has been used in the AOAC method (1) and the official Japanese method (2, 3) for determining organophosphorus pesticide residues in agricultural products, but it has seldom been used in an analytical method for chlorinated pesticide residues. Alessandro et al. use graphitized carbon black as an enrichment adsorbent material in the determination of chlorinated pesticides in water (4).

We found that chlorinated pesticides adsorbed on activated charcoal were retained even after the column was washed with a large volume of acetonitrile-water (60 + 40), but a small portion of fat extracted from milk was eluted. Therefore, we were able to separate chlorinated pesticides from fat in the determination of chlorinated pesticides in milk by use of acetonitrile-water (70 + 30) extraction and activated charcoal column chromatography.

In this paper we report on the use of activated charcoal for the recovery of chlorinated pesticides from milk.

METHOD

Reagents

(a) *Pesticide grade solvents.*—Acetonitrile, acetone, and hexane (Wako Pure Chemical Industry).

(b) *Activated charcoal.*—Coconut shell charcoal (Wako Pure Chemical Industry), 250–300 mesh. Wash with acetone and heat 3 h at 400°C.

(c) *Sodium carbonate solution.*—1%. Dissolve 10 g analytical reagent grade sodium carbonate in 1 L water.

(d) *Extracting solvent.*—Acetonitrile-water (70 + 30).

(e) *Eluting solvent.*—Acetone-hexane (1 + 1).

(f) *Pesticide standard solution.*—Standards (>95%) were obtained from Wako Pure Chemical Industry. Prepare acetone solution at the following levels (μg/mL): α-BHC 0.04, β-BHC 0.16, γ-BHC 0.04, δ-BHC 0.08, *p,p'*-DDE 0.05, *p,p'*-DDD 0.1, *p,p'*-DDT 0.2, and dieldrin 0.05.

Apparatus

(a) *Gas chromatograph.*—Yamaco Model G 2800EC (Yanagimoto Mfg Co. Ltd, Kyoto, Japan) equipped with ^{63}Ni source electron capture detector and 3 mm id × 2 m glass column packed with 2% DEGS + 0.5% H_3PO_4 on 60–80 mesh Chromosorb W DMCS (Gas Kuro Kogyo Ltd, Tokyo, Japan). Operating conditions: temperatures (°C)—column 200, injection port and detector 240; nitrogen carrier gas flow 80 mL/min.

(b) *Activated charcoal chromatographic column.*—15 mm id × 25 cm glass tube fitted with stopcock and 100 mL reservoir. Plug with small wad of cotton previously washed with benzene, and add 0.5 g activated charcoal with small portions of water; top with cotton wad.

Procedure

Measure 50 mL milk into 200 mL separatory funnel, add 100 mL acetonitrile, and shake vigorously 10 min on mechanical shaker. Mixture will separate into liquid and coagulated solid layers. Let stand 10 min and filter liquid extract

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Table 1. Adsorption (%) on 0.5 g activated charcoal of chlorinated pesticides from 200 mL acetonitrile–water at different ratios

Pesticide	Added, μg	Acetonitrile concn, v/v%				
		100	90	80	70	60
α-BHC	0.04	0	3	33	91	102
β-BHC	0.16	0	9	36	88	95
γ-BHC	0.04	0	34	74	95	104
δ-BHC	0.08	19	20	42	100	100
Dieldrin	0.05	7	21	55	100	100
p,p'-DDE	0.05	91	92	92	98	105
p,p'-DDT	0.2	73	100	100	100	100
p,p'-DDD	0.1	53	79	80	93	97

through paper into 300 mL Erlenmeyer flask. Extract solid layer twice with 30 mL acetonitrile–water (70 + 30) in original separatory funnel. Filter these 2 extracts through same paper used for first extract and combine extracts in same flask. Add 30 mL water to extract. After passing entire extract solution through activated charcoal chromatographic column, wash the column with additional 10 mL water and discard eluate. Add 100 mL acetone–hexane (1 + 1) to column and collect this fraction containing chlorinated pesticides. Transfer fraction to 500 mL separatory funnel containing 100 mL water. Separate hexane and aqueous layers by shaking 5 min on mechanical shaker. Re-extract aqueous layer with 50 mL hexane. Wash combined hexane extracts with 50 mL 1% sodium carbonate solution and then with 50 mL water by shaking 5 min. Dry hexane extract over anhydrous sodium sulfate and concentrate to 2–5 mL in Kuderna–Danish concentrator. Inject suitable aliquot of concentrate into gas chromatograph and compare retention times and peak heights of samples with those of standards for quantitation.

Results and Discussion

Milk usually consists of about 3% fat, 8% solids, and 89% water. When 100 mL acetonitrile is added to 50 mL milk, an acetonitrile–water (70 + 30) system may be formed. The milk emulsion is broken, and a homogeneous solution of acetonitrile–water–fat, containing the chlorinated pesticides, is cleanly separated from coagulated solids. Two extractions with acetonitrile–water (70 + 30) are necessary to avoid forming a solid cake and also extracting fat. Regardless, about 5% fat is extracted. Less than 70% acetonitrile in water was unable to separate the layers readily. The use of methanol or ethanol in place of acetonitrile did not lead to separate layers.

To confirm the adsorption efficiency of activated charcoal, we examined the percentage of chlorinated pesticides adsorbed on 0.5 g activated charcoal column for 200 mL acetonitrile–water, at different ratios, fortified with 1 mL pesticide standard solution. As shown in Table 1, 200 mL acetonitrile–water at ratios greater than 80 + 20 caused pesticides to elute; to obtain better and more reliable adsorption, acetonitrile concentration of eluting solvent must be decreased to less than 70%. In the present study, it was ad-

Table 2. Recovery (%) of chlorinated pesticides from fortified milk samples

Pesticide	Fortification level, μg	Recovery, mean ± SD[a]
α-BHC	0.04	99.8 ± 2.7
	0.08	98.0 ± 4.1
	0.4	98.2 ± 3.8
β-BHC	0.16	103.2 ± 3.4
	0.32	101.7 ± 2.8
	1.6	97.4 ± 5.1
γ-BHC	0.04	99.9 ± 2.8
	0.08	99.7 ± 3.5
	0.4	91.1 ± 3.1
δ-BHC	0.08	96.7 ± 2.6
	0.16	99.2 ± 5.2
	0.8	98.1 ± 5.9
Dieldrin	0.05	102.2 ± 3.2
	0.1	101.8 ± 3.0
	0.5	102.6 ± 4.7
p,p'-DDE	0.05	95.2 ± 4.5
	0.1	94.8 ± 3.4
	0.5	88.5 ± 4.5
p,p'-DDT	0.2	90.0 ± 4.0
	0.4	91.8 ± 6.3
	2	92.4 ± 5.4
p,p'-DDD	0.1	94.4 ± 3.4
	0.2	96.2 ± 2.3
	1	86.9 ± 3.0

[a] Based on 5 replicate analyses.

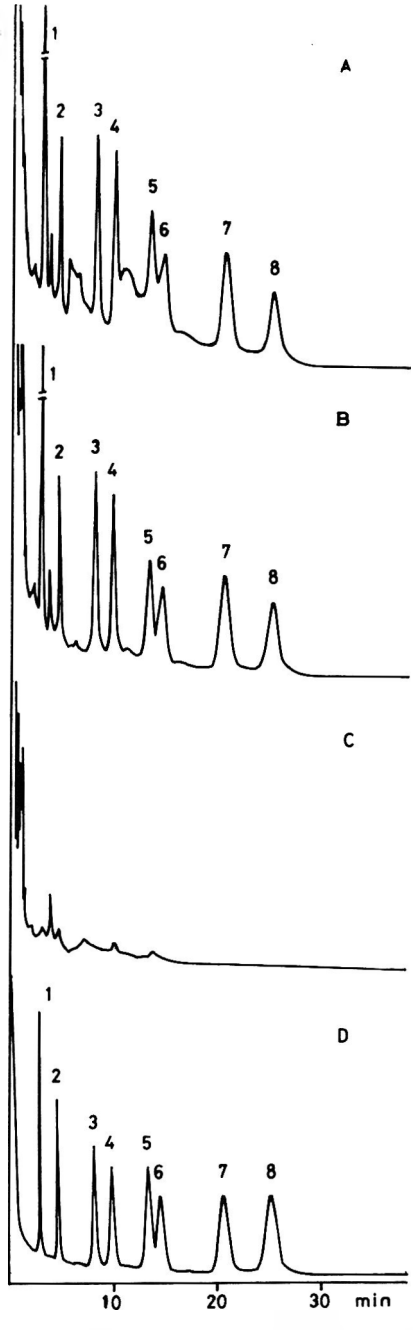

Figure 1. Gas chromatograms of A, milk fortified with 1 mL pesticide standard solution without alkaline washing; B, after alkaline washing; C, reagent blank from overall procedure; D, 1 μL pesticide standard solution. Peaks: 1, α-BHC; 2, γ-BHC; 3, p,p'-DDE; 4, dieldrin; 5, δ-BHC; 6, β-BHC; 7, p,p'-DDT; 8, p,p'-DDD.

Table 3. Comparative results of chlorinated pesticide residues found in a commercial milk sample by existing method and proposed method

Residue	Amount,[a] ppb	
	Existing method	Proposed method
α-BHC	1.13 ± 0.03	1.42 ± 0.21
γ-BHC	0.20 ± 0.02	0.25 ± 0.03
Dieldrin	0.66 ± 0.03	0.71 ± 0.05
p,p'-DDE	0.87 ± 0.05	1.39 ± 0.09

[a] Each value represents mean ± SD for 5 analyses.

justed to about 60%. The adsorption of charcoal activated at 400°C was constant and reproducible during 2 weeks at least. Activated charcoal must have a mesh size to obtain the stable adsorption activity and reproducible results. It was determined that particles smaller than 300 mesh are not retained by a plug of cotton.

Elution with 100 mL acetone–hexane (1 + 1) yielded good recoveries of chlorinated pesticides. Four % fat is in the eluate from the charcoal column, and 1% fat is in the acetone–hexane eluate.

Unknown organic compounds that eluted in a broad overlapping peak on the gas chromatogram were effectively excluded by washing the column eluate with alkaline solution (Figure 1). These compounds did not appear in the blank test of the overall procedure.

The recovery of chlorinated pesticides from actual milk samples was studied at 3 fortification levels. These results, compensated for background contaminations, are shown in Table 2. Background levels of α-BHC, γ-BHC, dieldrin, and p,p'-DDE are shown in Table 3. These average recoveries were satisfactory and reproducible.

For a 50 g sample, 2 mL final solution, and 1 μL injection, detection limits for the chlorinated pesticides are α-BHC 0.2, β-BHC 0.8, γ-BHC 0.2, δ-BHC 0.4, p,p'-DDE 0.3, p,p'-DDT 1, p,p'-DDD 0.5, and dieldrin 0.3 ppb.

This new method for commercial milk samples was compared with an existing method involving fat extraction, solvent partition, and Florisil column cleanup. The results obtained from the 2 methods were similar, as shown in Table 3. The present method can save time in extraction and concentration steps and does not use large volumes of solvents and as much glassware compared with the existing method; it is simple, rapid, and reliable for the routine determination of chlorinated pesticide residues. Finally, the

proposed method may be applicable to other sample types and to analysis of food additives and contaminants.

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Ion-Pair Reverse Phase Liquid Chromatographic Determination of Sodium Acifluorfen in Feed

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Ion-pair reverse phase liquid chromatography (LC) and UV detection at 280 nm have been used to determine sodium acifluorfen (sodium-5-[2-chloro-4-(trifluoromethyl)-phenoxy]-2-nitrobenzoate), an experimental diphenyl ether herbicide, in dog feed. Sodium-5-(2,4-dichlorophenoxy)-2-nitrobenzoate is used as the internal standard. The feed is homogenized in 0.01N HCl, followed by ethyl acetate extraction, and centrifugation. The organic layer is removed and evaporated and the residue is reconstituted in methanol and filtered before LC analysis (mobile phase methanol-water (58 + 42), 0.005M in tetrabutylammonium phosphate and 0.045M in $(NH_4)_2HPO_4$, at pH 7.4). The ion-pair technique offers a high degree of control over the retention characteristics of the herbicide and internal standard. The use of the internal standard permits precise and accurate quantitation and substantially reduces analysis time compared with the external standard method.

Sodium acifluorfen (sodium-5-[2-chloro-4-(trifluoromethyl)-phenoxy]-2-nitrobenzoate) is a selective herbicide used for pre- and post-emergence residual weed control of a wide spectrum of annual broadleaf weeds and grasses in soybeans, peanuts, and other large seeded legumes. Sodium acifluorfen is the active ingredient in Blazer® (Rohm and Haas, United States and Brazil), and in Tackle® (formerly owned by Mobil Oil Corp., now owned by Rhône-Poulenc Chemical Co.). Tackle is currently sold in Brazil and an experimental use permit has been granted for its use in the United States. Toxicity testing is under way for the purpose of obtaining full registration in the United States. To assist in the safety assessment program for Tackle, we developed a reliable liquid chromatography (LC) assay procedure to provide assurance of herbicide homogeneity and requisite concentration in dog feed.

The use of LC for determining sodium acifluorfen in feed was investigated as an alternative to the residue method reported by Adler et al. (Adler, I. L., Augenstein, L. L., & Rogerson, T. D. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 1456–1458), which specifies a gas chromatographic procedure. Early attempts used simple acid extraction procedures and conventional reverse phase LC techniques; however, the sodium acifluorfen peak could not be satisfactorily resolved from feed components in a reasonable time. Rather than incorporate more elaborate and time-consuming sample preparations to overcome this, an ion-pair LC technique was developed to effect the selectivity required for separation and analysis.

Received August 27, 1982. Accepted March 18, 1983.

METHOD

Reagents

(a) *Methanol and ethyl acetate.*—Baker Analyzed LC grade.

(b) *Water.*—Distilled and deionized.

(c) $(NH_4)_2HPO_4$.—Baker Analyzed.

(d) *Mobile phase.*—Methanol-water (58 + 42 v/v), 0.005M with respect to tetrabutylammonium phosphate (TBA) and 0.045M with respect to dibasic ammonium phosphate (pH of aqueous phase = 7.4). Filter aqueous solution and methanol through Whatman 0.45 μm discs and degas under reduced pressure before use.

(e) *Ion-pair reagent.*—Use as received. Tetrabutylammonium phosphate (Eastman Kodak Co., Rochester, NY 14650).

(f) *Standards.*—Both acifluorfen (99% purity) and internal standard (98% purity) were supplied by Mobil Chemical Co. as the free acids.

Apparatus

Liquid chromatograph.—Hewlett-Packard 1084B equipped with variable wavelength detector operated at 280 nm (sample) and 430 nm (reference). Chromatographic columns: 150 × 4.6 mm id, 5 μm particle diameter Altex C-18 reverse phase or 250 × 4.6 mm id, 5 μm particle diameter C-8 reverse phase DuPont Zorbax operated at 1.5 mL/min flow rate. Maintain column oven at 30°C. Instrument is equipped with variable volume autoinjector; typical injection volumes are 10–50 μL depending on concentration of sodium acifluorfen in feed.

Preparation of Feed Samples

Accurately weigh 1.00 g animal feed (Purina 5007 Dog Chow) in disposable plaster weighboat and transfer feed to 30 mL Corex centrifuge tube. Add 500 μL sodium-5-(2,4-dichlorophenoxy)-2-nitrobenzoate (internal standard) solution containing amount of material equal to nominal per gram concentration of sodium acifluorfen in feed. Add 10 mL 0.01 HCl to tube and homogenize on Polytron homogenizer for 30 s. Add 5–10 mL ethyl acetate to aqueous acid slurry and repeat homogenization for 30 s. Cover Corex tube with Parafilm and centrifuge at 3500 × *g*, for 10 min. Remove ethyl acetate layer with disposable Pasteur pipet and transfer to 13 × 100 mm disposable culture tube; evaporate ethyl acetate under gentle stream of nitrogen. Reconstitute residue in culture tube with 2 mL methanol and vortex-stir briefly. Filter reconstituted residue through disposable 0.2 μm disc (Gilman Acrodisc 4192). Transfer clear filtrate to appropriate septum-sealed LC vials for analysis.

Preparation of Standards

(**a**) *Sodium acifluorfen and internal standard stock solutions.*—Prepare 100 mL aqueous standard solutions of sodium acifluorfen and sodium-5-(2,4-dichlorophenoxy)-2-nitrobenzoate at each dosage level from the free acids. Accurately weigh sufficient amounts of acids to give final concentrations of sodium salts of 900, 600, and 40 mg/100 mL. Dissolve the acids in 25–50 mL water containing an equimolar amount of sodium hydroxide. Adjust final pH of solution to 8.5 with 1.0N HCl and 1.0N NaOH before filling flask (100 mL) to mark.

(**b**) *Sample standards.*—Accurately weigh 1.00 g control feed and transfer to 30 mL Corex centrifuge tube. Add equal amounts (500 μL) of sodium acifluorfen and internal standard at appropriate dosage level. Prepare sample in manner described above.

Calculations

Daily analyze sample standard data to determine correction factor reflecting deviation from the ideal, i.e., area sodium acifluorfen peak/area internal standard peak = 1.00, due to detector response, absorptivity, chemical selectivity, chemical purity, etc. Determine final concentration of sodium acifluorfen in feed using equation:

$$\text{SA, ppm} = (\text{area SA}/\text{area IS}) \times \text{CF} \times \text{dosage level, ppm}$$

where SA = sodium acifluorfen; IS = internal standard; CF = correction factor.

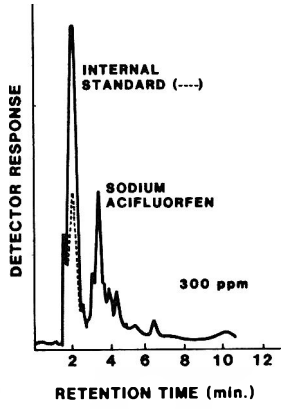

Figure 1. Conventional reverse phase LC chromatograms (methanol-water (58 + 42); Altex C-18) of feed extract containing 300 ppm sodium acifluorfen and internal standard (- - -).

Results and Discussion

The sample preparation procedure described consists of acidification of the feed to generate the free acid (acifluorfen) followed by extraction into ethyl acetate. Of the several different methods evaluated, the acid–ethyl acetate procedure was somewhat superior in terms of recovery and reproducibility. Initially, a gradient reverse phase LC method was used in the assay; however, the time required per analysis (approximately 20 min) proved impractical as the daily number of required analyses increased. Isocratic reverse phase LC (methanol–water (58 + 42)) showed little promise of being able to resolve the acifluorfen and internal standard peaks from the numerous feed component peaks in an acceptable time (Figure 1, 300 ppm feed fortification). Addition of TBA and buffer [$(NH_4)_2HPO_4$] to the mobile phase, however, allows a high degree of control over the retention volume of the reversible ion-pair complex formed between TBA and the acifluorfen (and internal standard) anion. When the same sample (Figure 1) is analyzed using a methanol-water (58 + 42), 0.005M TBA and 0.045M $(NH_4)_2HPO_4$ solvent system, the retention time is 7.0 min for the TBA–acifluorfen complex and 5.7 min for the TBA–internal standard complex. Figure 2 shows the ion-pair reverse phase LC chromatogram of the sample (300 ppm) and 2 other feed samples fortified at 4500 and 20 ppm. The acifluorfen peak and internal standard are well resolved from one another and from the extractable feed component peaks observed near the solvent peak. The internal standard, sodium-5-(2,4-dichlorophenoxy)-2-nitrobenzoate, was chosen because of its structural similarities

Table 1. Recoveries of sodium acifluorfen standards from fortified dog food

Assay No.	Spike level, ppm: 4500 Found, ppm	4500 Rec., %	300 Found, ppm	300 Rec., %	20 Found, ppm	20 Rec., %
1	3840	85	272	91	17.5	88
2	3870	86	258	86	17.2	86
3	3840	85	245	82	17.5	88
4	3870	86	258	86	17.4	87
Mean		86		86		87
SD		0.58		3.68		0.96
RSD, %		0.7		4.2		1.0

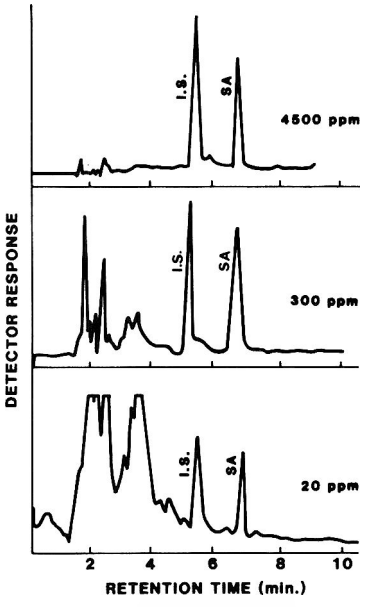

Figure 2. Ion-pair reverse phase LC chromatograms (methanol-water (58 + 42), 0.005M in TBA and 0.045M in $(NH_4)_2HPO_4$; Altex C-18) of food sample extracts containing sodium acifluorfen (SA) and internal standard (I.S.) at 4500, 300, and 20 ppm.

to sodium acifluorfen, and in particular, its comparable pKa (3.5). The separation observed in Figure 2 at a nominal mobile phase pH of 7.4 (i.e., complete ionization) was found to be both sufficient and practical in terms of the nature of the assay and the sample throughput. Recoveries of sodium acifluorfen using this procedure were evaluated at the 3 fortification levels discussed above. The results (Table 1) show average recoveries of 86% (4500 ppm), 86% (300 ppm), and 87% (20 ppm) with relative standard deviations of 0.7, 4.2, and 1.0%, respectively.

The assay has been used for the analysis of dog food samples from chronic and subchronic toxicity studies on sodium acifluorfen. Figure 2 shows representative chromatograms of dog food samples fortified at various levels from a subchronic study. A total of 156 samples and 26 blanks (0 ppm) were analyzed in duplicate. In addition, 23 sample standards were prepared at the 3 fortification levels (4500, 300, and 20 ppm) to generate the correction factor used in determining the sodium acifluorfen levels of the feed (see *Method* section). The correction factor represents the ratio of peak areas for sodium acifluorfen and internal standard fortified in the sample standards, and its standard deviation is a good measure of the overall precision of the assay. Table 2 includes the statistics for the correction factor from sample standards prepared at each dosage level during the study.

Table 2. Statistical analysis of correction factors derived from sample standards

Statistic	Fortification level, ppm: 20	300	4500
No. sample standards	8	8	7
Data points (injections)	26	50	22
Mean correction factor[a]	1.00	0.93	0.89
SD	0.05	0.05	0.05
Rel. SD, %	5.0	5.4	5.6

[a] Mean correction factor = area sodium acifluorfen/area internal standard peak for control feed samples fortified with equivalent amounts of the 2 compounds and prepared in a manner identical with actual samples.

Conclusion

Ion-pair reverse phase LC offers a high degree of control over the retention characteristics of the herbicide, sodium acifluorfen, and the internal standard, sodium-5-(2,4-dichlorophenoxy)-2-nitrobenzoate. This selectivity minimizes sample preparation before analysis, and combined with the use of the internal standard allows for a high degree of precision and accuracy in the assay.

Gas Chromatographic Determination of Trifluralin, Diallate, Triallate, Atrazine, Barban, Diclofop-Methyl, and Benzoylprop-Ethyl in Sediments at Parts Per Billion Levels

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A sensitive gas chromatographic method was developed for the multiresidue determination of 7 neutral herbicides in sediment. The sediment sample was ultrasonically extracted 3 times with acetone. The extract was concentrated to about 50 mL and then partitioned with 2% $KHCO_3$ and CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4. The concentrated extract was then cleaned up on a 10% deactivated Florisil column, and the herbicides were separated into 2 fractions. Further cleanup of these fractions was performed on activated Florisil columns. Electron capture detection was used for analysis except for atrazine which was analyzed by nitrogen-phosphorus detection. Recoveries of the herbicides from fortified sediment samples at 3 levels were generally better than 80%. The practical detection limit of this method was 1 ppb or 1 ng/g based on a 50 g sediment sample.

We recently reported a multiresidue method for the determination of 7 neutral herbicides in water (1). Using the "modular concept" of method development, this procedure was designed with the intention of incorporating several classes of organic pollutants including 10 acid herbicides.

In this report, we present a method for the analysis of the same 7 neutral herbicides (Table 1) in sediments. This method has also been designed to be extended later to the analysis of other neutral compounds such as polychlorinated biphenyls (PCBs), organochlorine pesticides, and chlorobenzenes.

Experimental

Apparatus

(a) *Gas chromatograph.*—(1) Model 5710A equipped with ^{63}Ni electron capture detector and Model 7672A automatic liquid sampler (Hewlett-Packard, Avondale, PA 19311). Operating conditions: injection port 200°C; column, see below; detector 300°C. Carrier gas argon-methane (95 + 5), flow rate 30 mL/min. (2) Model 5710A equipped with Model 18789A nitrogen-phosphorus-selective detector (NPD) (Hewlett-Packard). Operating conditions: injection port 200°C; column 185°C (3% OV-1); detector 300°C. Carrier gas helium, flow rate 30 mL/min. Detector gases: air, 50 mL/min; hydrogen, 3.0 mL/min. Collector voltage adjusted to give 50% FSD at 1 × 32 with electrometer zero off.

(b) *GC columns.*—1.8 m × 2 mm id coiled glass columns packed with 3% OV-1 on 100–120 mesh Gas-Chrom Q, 185°C (column 1); 3% OV-225 on 80–100 mesh Chromosorb W HP, 245°C (column 2); 1.5% OV-17 + 1.95% OV-210 on 100–120 mesh Gas-Chrom Q, 200°C (column 3) (Chromatographic Specialities Ltd, Brockville, Ontario, Canada K6V 5W1); and 80–100 mesh Ultrabond 20M, 200°C (column 4) (Ultra Scientific Inc., Hope, RI 02831).

(c) *Filtration apparatus.*—Modified coarse (70–100 μm) sintered glass funnel, 100 × 40 mm id, with standard taper joint and suction side-arm (original equipment supplied by Ace Glass Inc., Vineland, NJ 08360).

(d) *Evaporating apparatus.*—Buchi rotary evaporator with thermostated bath (Fisher Scientific Co., Ltd).

(e) *Chromatographic columns.*—(1) 500 × 20 mm id with coarse fritted disk and Teflon stopcock (Fisher Scientific Co., Ltd). (2) 230 × 5 mm id

Table 1. Common and chemical names of the 7 neutral herbicides

Common name	Chemical name
Trifluralin	α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl *p*-toluidine
Diallate	*S*-(2,3-dichloroallyl) diisopropylthiocarbamate
Triallate	*S*-(2,3,3-trichloroallyl) diisopropylthiocarbamate
Atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine
Barban	4-chloro-2-butynyl *m*-chlorocarbanilate
Diclofop-methyl	methyl 2[4-(2',4'-dichlorophenyl)-phenoxy]propionate
Benzoylprop-ethyl	ethyl 2-(*N*-benzoyl-3,4-dichloroanilino)propionate

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Table 2. Single operator precision and recovery of 7 neutral herbicides in fortified sediments

Herbicide	Level (ng/g)	Replicates	Mean % recovery ± SD
Trifluralin	10	3	93.9 ± 3.2[a]
	1	12	96.1 ± 2.6
	0.1	6	130.2 ± 11.0
Diallate	200	3	86.6 ± 2.3[a]
	20	12	94.9 ± 5.1
	2	6	87.9 ± 13.9
Triallate	20	3	91.9 ± 1.6[a]
	2	12	98.3 ± 4.3
	0.2	6	120.4 ± 11.4
Atrazine	400	3	44.9 ± 2.5[a]
	40	12	99.1 ± 4.2
	4	5	84.2 ± 4.0
Barban	200	3	101.1 ± 2.1[a]
	20	12	98.6 ± 7.9
	2	5	104.0 ± 10.1
Diclofop-methyl	100	3	79.4 ± 1.0[a]
	10	12	99.1 ± 5.9
	1	5	58.4 ± 13.1
Benzoylprop-ethyl	50	3	92.4 ± 3.0[a]
	5	12	102.4 ± 5.4
	0.5	5	68.2 ± 14.2

[a] Samples acidified to pH ≤1 before extraction.

Pasteur pipet plugged at bottom with piece of silanized glass wool.

Reagents

Use pesticide grade hexane or petroleum ether (30–60°C), acetone, dichloromethane, isooctane, and benzene. Check each batch of solvents and chemicals for interferences before use.

(a) *Pure water.*—Prepare according to ref. 1.

(b) *Sodium sulfate.*—Anhydrous reagent grade (BDH Chemicals, Toronto, Ontario, Canada M8Z 1K5). Heat 18 h at 650°C and store in all-glass containers.

(c) *Metallic mercury.*—AnalaR grade (BDH Chemicals).

(d) *2% $KHCO_3$ solution.*—Dissolve ACS grade $KHCO_3$ (20 g) in pure water and dilute to 1 L.

(e) *Florisil.*—Florisil PR, 60–100 mesh, calcined at 650°C (factory-treated) and kept at 130°C until use (Supelco Inc., Bellefonte, PA 16823). *10% deactivated Florisil.*—Add 10 g pure water to 90 g activated Florisil. Mix well by tumbling (18 h) in tightly capped glass container before use. Prepare fresh weekly.

(f) *Herbicide standards.*—Analytical grade (98 + % pure). Obtained from manufacturers or U.S. Environmental Protection Agency (HERL, Research Triangle Park, NC 27711) and used as received. Prepare all stock solutions in benzene, working standards in isooctane-acetone (99 + 1), and spiking solutions in acetone.

(g) *Standard solutions.*—Prepare 2 mixed standards (solutions A and B) in isooctane–acetone (99 + 1) for GC calibration according to following table:

Soln	Herbicide	Concn, pg/μL
A	trifluralin	5
	diallate	100
	triallate	10
B	atrazine	100
	barban	100
	diclofop-methyl	50
	benzoylprop-ethyl	25

(h) *Fortification of sediment samples.*—Add 100 μL of an appropriate herbicide spiking solution in acetone to 50 g wet sediment in beaker to give herbicide concentrations equal to those listed in Table 2. Mix well with a spatula, cover beaker with aluminum foil, and keep at −10°C overnight before extraction. All sediment samples were obtained from various sites of Battle River, Old Man River, and Red Deer River in western Canada. These sediments, although high in organic content in some cases, have no detectable amounts of the 7 herbicides.

Sample Handling

Keep sediment samples frozen in dark in precleaned metal can immediately upon collection. The stability of these 7 herbicides in sedi-

ments has not been thoroughly studied as yet. On receipt of samples, perform extractions as soon as possible, and store extract at 4°C in dark until ready for cleanup and analysis.

Sample Preparation

Before extraction, mix and stir sample thoroughly. For naturally contaminated sediments, blend whole sample in 5 L stainless steel blender for 10 min at medium to high speed, with intermittent cooling to maintain the temperature of sample at or below room temperature. Subsample a 50 g aliquot for extraction.

Homogenization of wet sediment has been investigated for PCBs (2) but not extensively studied for other parameters. Almost all sediments we have dealt with have contained no large debris. If debris is present, it should be removed by passing the sample through a coarse sieve (1–2 mm).

Extraction

Weigh 50 g homogeneous wet sediment into 250 mL beaker. Weigh another representative sample (10–20 g) into tared beaker and determine moisture content by drying sample to constant weight at 105°C.

If necessary, wet sample in beaker with organic-free water to estimated 30% moisture content and mix thoroughly.

Add 100 mL acetone to sediment sample. Place ultrasonic cell disruptor horn so that it is immersed 2.0 cm into the suspension. Activate sonicator for 3 min in pulsed mode at 50% duty cycle with output control set to 8. Caution: To avoid losses of volatile components, keep sample below 45°C. Let sediment settle. Prepare 5 cm Celite 545 column in sintered glass filter funnel connected to round-bottom flask. Wash column with 100 mL acetone using vacuum suction from water aspirator. Discard washing. Decant supernatant liquid into Celite column as much as possible, and apply vacuum to collect extract into clean round-bottom flask. Repeat extraction twice with two 100 mL portions of acetone. Combine extract into same round-bottom flask.

After last filtration, transfer entire sediment sample to Celite filter. Apply vacuum until column is nearly dry. Rinse beaker with two 10 mL portions of acetone, filter through Celite column, and collect filtrate as before. Evaporate combined acetone extract to ca 50 mL with rotary evaporator under reduced pressure at ≤40°C.

Quantitatively transfer concentrated extract from flask into a 500 mL separatory funnel containing 100 mL 2% $KHCO_3$ solution. Rinse flask with two 50 mL portions of dichloromethane and transfer rinsings to the 500 mL separatory funnel. Shake funnel 1 min and vent frequently. Let layers separate. Drain lower organic layer through 5 cm (ca 80 g) anhydrous Na_2SO_4 in coarse sintered glass filter column. Apply suction and collect filtrate in 500 mL round-bottom flask. Repeat extraction of slightly basic aqueous layer with 50 mL methylene chloride. Discard aqueous layer. Rinse Na_2SO_4 column with additional 30 mL methylene chloride. Collect filtrate in the round-bottom flask.

To combined filtrates, add 3 mL isooctane. Evaporate extract to ca 10 mL on rotary evaporator at ≤40°C as before. Add 50 mL hexane to concentrated extract and carefully evaporate to 3–5 mL.

Cleanup

Prepare cleanup column by adding 20 g 10% deactivated Florisil into chromatographic column. Tap column gently to settle adsorbent. Add 1 cm anhydrous Na_2SO_4 to top of column. Prewet column with 100 mL hexane and let solvent drain just to top of Na_2SO_4 layer. Discard hexane.

Using Pasteur pipet, quantitatively transfer previous concentrated extract, plus three 5 mL hexane rinsings, to Florisil column. When extract just enters into Na_2SO_4 layer, elute column with 200 mL 25% benzene in hexane (Fraction A). Collect eluate in a 500 mL round-bottom flask. Add 3 mL isooctane and evaporate to ca 3 mL in rotary evaporator at 40°C. Fraction A contains trifluralin, diallate, and triallate.

Elute same column with 200 mL 1% methanol in benzene (Fraction B). Add 3 mL isooctane to eluate and evaporate to ca 3 mL as above. Fraction B contains atrazine, barban, diclofop-methyl, and benzoylprop-ethyl.

Fractionation

To further clean Fraction A, prepare column by filling with 20 g activated Florisil followed by 1 cm anhydrous Na_2SO_4. Wash column with hexane as described above.

Quantitatively transfer concentrated extract from Fraction A onto column. Rinse flask with two 1 mL portions of 20% methylene chloride in hexane. Elute column with 200 mL of same solvent to obtain Fraction 1. Discard this fraction or save for analysis of organochlorine pesticides, PCBs, and chlorobenzenes.

Elute same column with 250 mL methylene chloride to collect Fraction 2. Add 3 mL isooctane and evaporate to ca 10 mL in rotary evapo-

rator as before. Add 50 mL hexane and carefully evaporate to ca 3 mL. Make up to 10 mL in centrifuge tube. Fraction 2 contains trifluralin, diallate, and triallate. Shake this fraction with mercury until metal is shiny to remove sulfur compounds. Analyze by electron capture (EC)-GC.

To further clean Fraction B, prepare microcolumn by plugging 20 cm long Pasteur pipet with piece of silanized glass wool. Fill pipet with 4 cm activated Florisil (with gentle tapping) followed by 0.5 cm anhydrous Na_2SO_4.

Prewet micro Florisil column with 2 mL 25% benzene in hexane (v/v). After solvent has drained just to top of column, quantitatively transfer Fraction B to column. Elute column with 10 mL 25% benzene in hexane and discard eluate. Then elute column with 1.5% acetone in benzene to collect 10 mL eluate in centrifuge tube. This is Fraction 3 and contains atrazine, barban, diclofop-methyl, and benzoylprop-ethyl.

GC Analysis

Analyze both fractions (2 and 3) by EC-GC except for atrazine. Analyze atrazine in Fraction 3 by using a nitrogen-phosphorus-selective detector.

Results and Discussion

Extraction

Initially, we intended that the extraction procedure for the 7 neutral herbicides would incorporate the 10 acid herbicides (3) to develop a multiclass multiresidue method. Thus the sediment was acidified to ≤ pH 1 before extraction so that both types of herbicides could be extracted simultaneously. However, several disadvantages were observed with this procedure: (*1*) Atrazine was poorly recovered (about 45%, see Table 2). (*2*) Emulsions formed with sediment extracts of high humus content during the base partitioning step, causing low recovery of all 7 herbicides. (*3*) Sample co-extractives increased considerably. While these sediment co-extractives had minor effects on the analysis of the less polar neutral herbicides, namely, trifluralin, diallate, and triallate (Fraction 2), some co-extractives were eluted in Fraction 3 and caused considerable interferences in the EC-GC analysis for barban, diclofop-methyl, and benzoylprop-ethyl at low levels. Since quantitative recoveries of these neutral herbicides required no acidification, all subsequent spiked recovery experiments were carried out without acidification of sample. Although it is not feasible to incorporate the acid herbicides into the present scheme, the procedure does appear to be compatible with the extraction and cleanup of several other classes of neutral compounds, namely, PCBs, 18 organochlorine insecticides, and chlorobenzenes.

Sample Cleanup

Although a rigorous cleanup procedure is proposed here to meet the analytical requirements at the detection limit, it is not necessary to go through all the cleanup steps under less demanding situations such as sediments with less co-extractives or at higher contamination levels. For sediments contaminated with these herbicides at low ppm levels, we have found that only a base partitioning cleanup is required. At 10–100 ppb levels or higher, a partitioning step together with a 10% deactivated Florisil column cleanup are generally sufficient to remove sample co-extractives.

It should be noted that, in the cleanup of Fraction A by means of a macro activated Florisil column, not all of the common organochlorine insecticides will be removed in the methylene chloride–hexane (20 + 80) fraction. Dieldrin, α- and β-endosulfans, endrin, and p,p'-methoxychlor, if present, will be co-eluted in the trifluralin, diallate, and triallate fraction. However, the presence of these 5 organochlorines would not interfere with the GC analysis of the neutral herbicides because the latter have much shorter retention times.

GC Analysis

For direct analysis of barban without derivatization, the GC column should be primed by repetitive injections of the standard. In routine analysis, sample injections should be interspersed with standard injections. (See also discussion in Ref. 1.)

The 3% OV-1 column is used for daily analysis of the herbicides, whereas the Ultrabond 20M column is recommended for confirmation purposes. Reference 1 shows the retention times of the 7 herbicides on 4 GC columns including the two above and a typical chromatogram of the 7 neutral herbicides on the 3% OV-1 column.

Precision, Recoveries, and Detection Limits

The precision and recoveries of 7 neutral herbicides from fortified sediment samples at 3 concentration levels are summarized in Table 2. Fortification was carried out by adding known

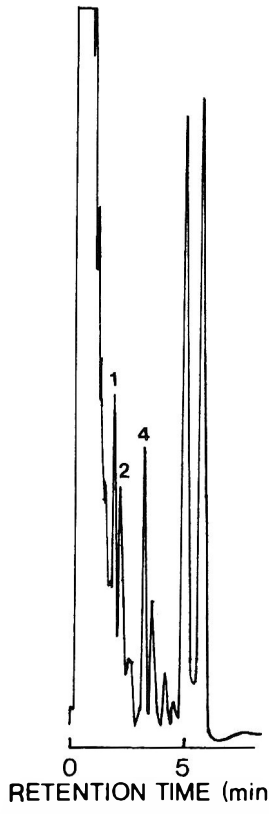

Figure 1. Gas chromatogram (ECD) of fraction 2 on 3% OV-1 column at 185°C. Extract was derived from sediment sample fortified at detection limit. Injection volume: 10 μL, attenuation ×16. Peak identification: (1) trifluralin, (2) diallate, and (4) triallate.

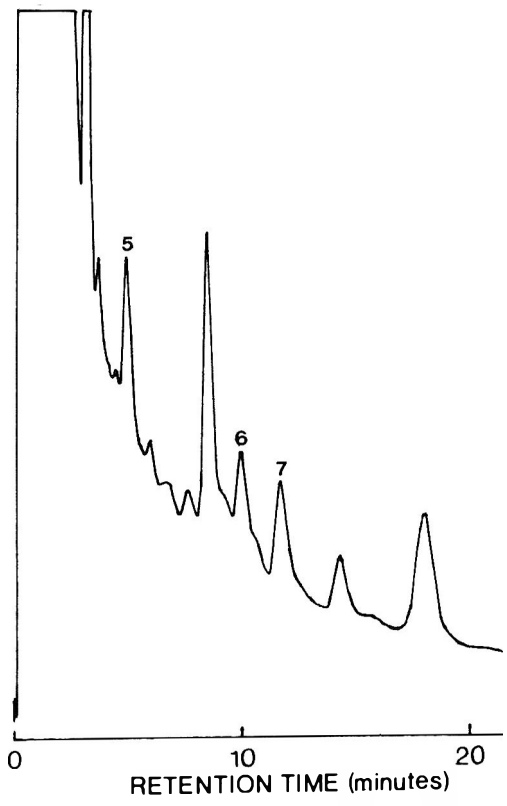

Figure 2. Gas chromatogram (ECD) of fraction 3 on 3% OV-1 column at 200°C. Extract was derived from sediment sample fortified at detection limit. Injection volume: 10 μL, attenuation ×16. Peak identification: (5) barban, (6) diclofop-methyl, and (7) benzoylprop-ethyl. Note that atrazine (3) in this fraction could only be detected by NPD at this level.

amounts of standard solution onto the sediments and equilibrating samples overnight at 4°C before extraction. In most cases, recoveries of herbicides were better than 80%. However, lower recoveries (60–70%) were experienced for diclofop-methyl and benzoylprop-ethyl near detection limits. Also, if the sediment sample is acidified to pH ≤1, recovery of atrazine is low. In this work, 3 types of river sediments from prairie provinces were used, namely from Old Man River, Red Deer River, Battle River. The sediments varied from grey to dark grey indicating various amounts of organic matter. They also varied from sandy loam to silty clay in texture.

The practical detection limit of the herbicides in sediments was set at 1 ng/g (ppb), based on a 50 g sediment sample with a 30% moisture content.

Representative chromatograms of extracts from sediment samples spiked at or near the detection limit after all necessary cleanup are shown in Figures 1 and 2. Control (blank) sample chromatograms exhibited no spurious peaks with the herbicides after the sample had been carried through the cleanup steps. For further discussions on the analysis of these neutral herbicides see Ref. 1.

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Gas Chromatographic Determination of Maleic Hydrazide Residues in Potato Tubers

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A gas chromatographic (GC) method is described for the determination of maleic hydrazide residues in potato tubers by oxidation of maleic hydrazide with aqueous lead dioxide to 3,6-pyridazinedione in the presence of cyclopentadiene. The reaction product, a volatile Diels-Alder adduct, could be detected in potatoes at levels in excess of 0.05 ppm with an electron capture detector. Recoveries of maleic hydrazide (as the Diels-Alder adduct) from potatoes at fortification levels of 0.1 to 10 ppm averaged 91.7%.

Maleic hydrazide (1,2-dihydro-3,6-pyridazinedione), a systemic growth regulator, is applied to potatoes as a sprout inhibitor, usually as the diethanolamine salt. The diethanolamine salt is considered toxic to humans, but on foliar application, the diethanolamine fraction is rendered harmless through oxidation during absorption and translocation to the tubers (1). Newsome (2) demonstrated that maleic hydrazide, once translocated, remains intact within the tuber and is not readily metabolized or conjugated.

Renewed concern with regard to the mutagenic potential of maleic hydrazide (3, 4) has emphasized the need for concise, sensitive methods to monitor residues in food crops. The polar nature of maleic hydrazide severely limits methods that can be used for its isolation and analysis. Most specific analytical methods (5–7) require prior column chromatographic cleanup to avoid detector interferences. This paper describes a quantitative gas chromatographic (GC) method which avoids involved cleanup procedures by using a unique selective derivatization reaction for maleic hydrazide. The electron capture (EC) detector is highly sensitive to the derivative, and detector interferences from contaminants are minimal.

METHOD

Apparatus

(a) *Mass spectrometer.*—Perkin-Elmer Hitachi.

(b) *Gas chromatograph.*—Tracor Model 222 equipped with electron-capture ^{63}Ni detector (with linearizer), Perkin-Elmer nitrogen-phosphorus detector, 1.8 m × 4 mm id glass column packed with 2% OV-275 on 80–100 mesh HP Chromosorb W. Operating parameters: injection port 230°C; column 210°C; detector 300°C; helium carrier gas flow rate 60 mL/min. Under these conditions, the 3,6-pyridazinedione–cyclopentadiene adduct (IV) had a retention time of 7.3 min.

(c) *Integrator.*—Hewlett-Packard 3380 A.

(d) *Melting point apparatus.*—Kofler hot-stage microscope.

(e) *Spectrophotometer.*—Beckman IR-20A.

Reagents

(a) *1,3-Butadiene.*—CP grade (Matheson of Canada, Whitby, Ontario).

(b) *Cyclopentadiene.*—Prepared (8) as required by depolymerization of technical dicyclopentadiene purchased from J.T. Baker Chemical Co., Toronto, Ontario, Canada.

(c) *Lead tetraacetate.*—BDH Chemicals, Halifax, Nova Scotia, Canada.

(d) *Maleic hydrazide.*—Aldrich Chemical Co. Inc., Milwaukee, WI.

(e) *3,6-Pyridazinedione–1,3-butadiene adduct.*—Prepared as described by Clement (9). mp 156–158°C [lit. (9) mp 157–159°C]; IR (Nujol) 1645 cm^{-1} (c = 0); mass spectrum m/z 164.

(f) *3,6-Pyridazinedione–cyclopentadiene adduct.*—Prepared initially by Clement's method. mp 182–184°C [lit. (10) mp 183–184°C]; IR (Nujol) 1643 cm^{-1} (c = 0); mass spectrum m/z 176.

Procedure

Homogenize samples of diced potatoes (40 g) with methanol (125 mL) in Waring blender for 2 min and filter homogenate through Whatman No. 1 paper. Rinse blender vessel with additional methanol, (50 mL) and filter rinse. Dilute combined filtrate to exactly 200 mL, place 5 mL aliquot (equivalent to 1.0 g sample) in 50 mL round-bottom flask, and remove methanol on

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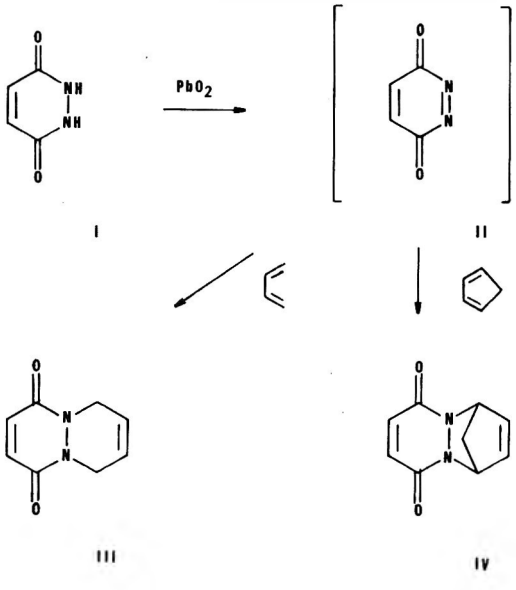
rotary evaporator (water bath at ca 35°C). Add 15 mL chloroform containing 10 μL cyclopentadiene and initiate vigorous stirring at room temperature. (Freshly distilled cyclopentadiene is preferable for low levels (≤0.5 ppm) of maleic hydrazide where detector interferences from polymeric impurities may result.) Immediately add suspension of freshly precipitated lead dioxide prepared by adding 25 mg lead tetraacetate to 5 mL water. Continue stirring for 5 min, then decant chloroform–water mixture into 60 or 125 mL separatory funnel. Rinse flask with 10 mL chloroform and add rinse to separatory funnel. Shake, let settle, and remove chloroform layer. Repeat extraction with 10 mL chloroform. Reduce chloroform extracts to dryness on rotary evaporator. Add ca 2 mL benzene to residue and again reduce to dryness (this operation alleviates detector interferences from traces of chloroform). Add 1 mL benzene to residue and inject 10 μL aliquots into gas chromatograph for analysis. Prepare standard solutions of 3,6-pyridazinedione-cyclopentadiene adduct (IV), and calculate maleic hydrazide content of sample by multiplying adduct content by mol. wt maleic hydrazide/mol. wt adduct.

Results and Discussion

Previous characterization of the products from reaction of maleic hydrazide (I) with diazomethane (11) demonstrated that derivatives which retain maleic hydrazide in an enedione form are extremely sensitive to electron capture detection. Incorporation of this concept with a derivatization reaction uniquely specific to maleic hydrazide formed the initial basis for our investigations. The most appropriate sequence outlined in the literature (9) involved initial oxidation of maleic hydrazide with lead tetraacetate in acetic acid–dichloromethane solution. The resultant diazoquinone (II) is unstable, but in the presence of butadiene as a trapping agent, a volatile and thermally stable Diels-Alder adduct (III) is formed. This derivative had excellent GC characteristics and could be readily detected in nanogram quantities by electron capture detection. Further investigation of the reaction on microgram levels demonstrated that it normally gave moderate yields (52–65%) of the one major product. Somewhat higher (64–72%) and more consistent yields were achieved with the more reactive dienophile cyclopentadiene which gave the Diels-Alder adduct IV. Compound IV also had excellent GC characteristics but EC detection was somewhat less sensitive than to compound III.



When either of the above reactions was applied to dried potato tuber extracts spiked with maleic hydrazide, recoveries in the form of the corresponding derivatives decreased dramatically. Apparent losses corresponded with an increase in tuber extract content. Adherence of maleic hydrazide to nonaqueous insoluble material in the tuber extract was thought to be responsible. Previous indications (10) that the 3,6-pyridazinedione was nominally stable in polar solvents encouraged us to attempt the oxidation stage of the reaction in an aqueous medium. Lead tetraacetate was effectively eliminated as an appropriate oxidant because it rapidly decomposes to lead dioxide (PbO_2) on contact with water. The knowledge that PbO_2 had previously been used for the production of hydrazyl radicals from azanes (12) prompted an investigation of its potential as a substitute oxidant. Subsequently, a 2-phase procedure involving addition of freshly precipitated lead dioxide to a stirred mixture of cyclopentadiene in chloroform and maleic hydrazide in water gave the Diels-Alder adduct (IV) in near quantitative yield. Presumably, the diazoquinone (II) when formed (by oxidation of maleic hydrazide with suspended PbO_2) is quickly distributed into the chloroform layer where it is protected from reactions other than the desired Diels-Alder reaction. Potato tuber extracts fortified with maleic hydrazide were subjected to these new conditions, and recoveries as the Diels-Alder adduct were carried out on duplicate samples with quantitation by peak area. Recoveries at fortification levels of 0.1, 0.5, 1.0, 5.0, and 10.0 ppm

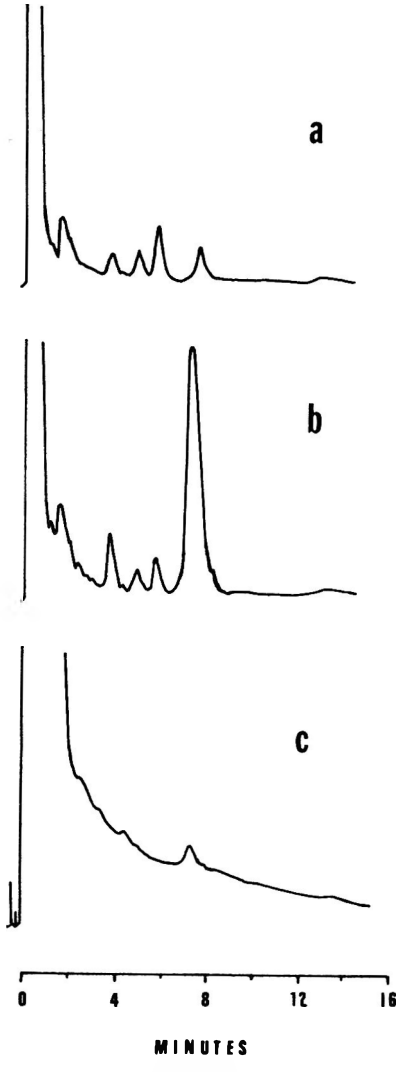


Figure 1. Gas chromatograms of potato tuber samples: a, 0 ppm maleic hydrazide (EC detector); b, 1 ppm maleic hydrazide (EC detector); c, 1 ppm maleic hydrazide (NP detector).

averaged 95.7, 94.0, 91.5, 87.9, and 90.4%, respectively, with a mean recovery of 91.7% and a coefficient of variation of 3.1%. Extracts equivalent to 1 g samples were used at each level. Due to the selective derivatization step, detector interferences from derivatized contaminants were kept to a minimum (Figure 1). A limit of sensitivity for detection of maleic hydrazide of 0.05 ppm was easily attainable. Because the electron capture detector is nonspecific, confirmation of positive maleic hydrazide determinations using an element-sensitive detector were also investigated. We found that maleic hydrazide derivative IV gave a nitrogen-phosphorus detector response equivalent to approximately $^1/_{10}$ that of the EC detector (Figure 1). In this manner, the presence of high maleic hydrazide levels (>0.5 ppm) could be further substantiated.

Field-treated potatoes (6.5 pints maleic hydrazide diethanolamine salt in 100 gal. water per acre) were analyzed after 20 weeks of storage at 55°C, and contained an average of 2.5 ppm maleic hydrazide. GC–mass spectrometry was used to confirm that the GC peak was due to the maleic hydrazide derivative. The mass spectral fragmentation pattern exhibited prominent ions at m/z 176 (67.2), 82 (100), and 66 (72.0).

The GC method developed for the determination of maleic hydrazide in potatoes is concise and specific, and should be readily applicable to other crops such as turnips, beets, carrots, and possibly tobacco.

Acknowledgment

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Evaluation of Nielsen-Kryger Steam Distillation Technique for Recovery of Phenols from Soil

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The Nielsen-Kryger steam distillation technique has been evaluated for recovery of phenols from soil. Soils are acidified to pH <1 and steam-distilled, and chlorinated phenols are extracted with toluene-methylene chloride (95 + 5). Mean recoveries from 4 samples spiked at 1 μg/50 g clay soil ranged from 67 to 78%. The method has limited utility for the recovery of other substituted phenols on the Environmental Protection Agency priority pollutant list.

Chlorinated phenols are extensively used as herbicides, insecticides, fungicides, antiseptics, and disinfectants. The annual production of pentachlorophenol (PCP) in the United States alone has been reported to exceed 40 000 tons (1). About 90–95% of this amount is used as a wood preservative. 2-Chlorophenol is used mainly for further chlorination to 2,4-dichlorophenol, 2,4,6-trichlorophenol (TCP), and PCP. 2,4,6-TCP is used as a fungicide and bactericide. 2,4-Dichlorophenol and 2,4,5-TCP are used as starting materials for the manufacture of pesticides, including 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), or herbicides, including 2(2,4,5-trichlorophenoxy)propionic acid (silvex).

The extensive use of chlorinated phenols is a potential source of serious environmental contamination. PCP is toxic, not only to wood-destroying organisms, but also to humans and other animals (2,3). 2,4,5-TCP is a precursor of the extremely toxic 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin.

The primary sources of PCP in soil are through its application as a herbicide and leaching from preservative-treated wood. 2,4,5-TCP appears in the soil through the degradation of 2,4,5-T or silvex. Similarly, 2,4-D degrades to 2,4-dichlorophenol. Washout from the atmosphere also contributes to soil contamination by chlorophenols.

Several analytical methods for isolating chlorinated phenols from water, soil, and biological tissues have been reported (4–7). The most common techniques for soil samples have involved 3 steps: extraction with sodium or potassium hydroxide, acidification of the extract, and further extraction from the acidic solution with an organic solvent (4). However, acidification of the basic extract to pH <6 sometimes produces a gel, which makes extraction with an organic solvent very difficult. Treatment of the extract with an ion-exchange resin eliminates some of these difficulties (8), and Soxhlet extraction, shaking with an organic solvent, or sonication (9), gives good recoveries. But these procedures also extract lipids, pigments, waxes, and other high molecular weight organics. Extensive column chromatographic cleanup of the extract is often necessary for trace analysis.

A steam distillation procedure for separation of organics, based on their vapor pressures and water solubilities, could provide a simple, convenient way to isolate chlorinated or other substituted phenols from soil samples (10). Recently, Kan et al. (11) reported the use of a modified Nielsen–Kryger steam distillation apparatus (10) to recover 2,4-dichlorophenol, a degradation product of 2,4-D butoxyethanol ester, from soil. There seems to have been no effort to apply this technique for the recovery of phenols in general from soil.

In our laboratory, the Nielsen-Kryger apparatus has been used extensively since 1978 to recover a large number of organic contaminants from soil. To date, more than 3000 soil samples have been analyzed by this method.

In the present study, we have also evaluated the Nielsen-Kryger steam distillation technique for determination of the Environmental Protection Agency (EPA) priority pollutants in soil. The procedure is obviously not efficient for analysis of compounds with significant water solubility. Such compounds exhibit poor volatility with steam. In addition, the continuous passage of warm water through the extracting solvent tends to scavenge water-soluble compounds back into the aqueous phase and thus back into the boiling flask. In the present work, this effect has been minimized by optimizing the extracting solvent.

Experimental

Apparatus

(a) *Gas chromatograph.*—Varian 3700 gas

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chromatograph, equipped with linearized ^{63}Ni electron capture and flame ionization detectors, and Varian 8000 Autosampler were used with Spectra Physics SP 4100 data system. Columns were fused silica SE 54, 30 m × 0.25 mm id, film thickness 0.25 μm (J. W. Scientific, Rancho Cardova, CA); and 1% SP 1240DA on 100–200 mesh Supelcoport, packed in a 1.8 m × 2 mm id glass column. Column temperatures: for chlorophenols, 100–200°C, initial hold 12 min, 8°/min to 200°C; for substituted phenols, 50–160°C, initial hold 2 min, 8°/min to 160°C. Injector temperature 200°C; detector temperature 320°C; nitrogen carrier gas. Flow rates: capillary column 2 mL/min; capillary make-up 30 mL/min; packed column 30 mL/min. Chart speed 1 cm/min.

(b) *Steam distillation apparatus.*—The modified design of Veith and Kiwus (10) (Ace Glass, Vineland, NJ) was used.

(c) *Shaker.*—Burrell wrist-action (Burrell Corp., Pittsburgh, PA).

Reagents

(a) *Solvents.*—All solvents were pesticide grade (MC & B, Norwood, OH).

(b) *Chemical standards.*—Analytical standards were purchased from Aldrich Chemical Co., Milwaukee, WI. Stock standards were prepared in acetone and working standards in hexane.

Soil Samples

(a) *Clay soil.*—A number of clay soil samples were collected from the Niagara Falls, NY area. These were homogenized together and stored at 4°C in glass jars. The mixture had a moisture content of about 25%. A blank run showed none of the phenols under investigation.

(b) *Potting soil.*—Potting soil (Hyper-Humus Co., Newton, NJ) was purchased locally.

Procedure

(a) *Spiking of standards.*—A 50 g portion of homogenized clay or potting soil was placed in a 2 L round-bottom flask and cooled in an ice bath. An appropriate phenol mixed standard (1 mL) was then added dropwise with shaking to the soil sample. To distribute the spiked standard uniformly, the flask was stoppered and swirled for another 2–3 min. It was then allowed to stand overnight in a refrigerator.

(b) *Extraction.*—Distilled water (500 mL) was added to the flask, followed by 40 mL sulfuric acid–water (1 + 1). A magnetic stirrer bar was introduced into the flask, and a Nielsen–Kryger distillation column was attached to it. The mixture was stirred at ambient temperature for 30 min, after which 10 mL toluene–methylene chloride (95 + 5) was introduced into the condenser. The flask was refluxed gently for 2 h and cooled, and the small amount of water trapped in the distillation column was drained. The organic layer was then passed through a small amount of washed anhydrous sodium sulfate into a 15 mL centrifuge tube. The walls of the column and the sodium sulfate were rinsed with a small amount of hexane. The combined organic layer was concentrated to ca 5 mL under a gentle stream of dry nitrogen. Most of the toluene was displaced with hexane. To remove the sulfurous material the concentrate was shaken with 2–3 drops of metallic mercury for 30 min.

(c) *Chromatography.*—The extract containing chlorophenols was analyzed on fused silica SE 54 and 1% SP 1240DA columns with an electron capture detector. The extract of other substituted phenols was analyzed on the 1% SP 1240DA column with a flame ionization detector. The peak areas were measured with Spectra Physics SP 4100 data system.

Results and Discussion

Initially, the recovery experiments were carried out by spiking the potting soil, which is rich in organic material, with 2,4,5-TCP. The mean recovery from 1 μg/50 g soil was 62 ± 8% ($n = 4$). Subsequently, detailed recovery experiments were carried out on a clay soil homogenate. Mean recoveries from soil spiked with various concentrations of chlorinated phenols are shown in Table 1. 3,4,5-TCP was recovered only in trace amounts.

The organic solvent and pH of the slurry were critical factors affecting recovery. Due to the polar nature of chlorophenols and because only a small amount of solvent (about 15 mL) is used, it is essential that the phenols be highly soluble in the extracting solvents. The technique also requires that the solvent mixture be water-immiscible, be lighter than water, and, to prevent solvent loss, have a high boiling point. Toluene–methylene chloride (95 + 5) proved to be the most suitable solvent. Satisfactory results were obtained only when the medium was acidified to pH <1 with sulfuric acid–water (1 + 1). This requirement can be attributed to the fact that phenols are acidic in nature and that at higher pH they are not freed from the soil matrix to be volatile in steam.

Our attempts to isolate phenols on the EPA Priority Pollutant list (Table 2) by this technique

Table 1. Recovery (%) of chlorinated phenols from 50 g soil [a]

Chlorophenol	Added, μg		
	10	1	0.1
	Clay Soil		
PCP	71 ± 3	70 ± 5	58 ± 5
2,3,4-TCP	87 ± 8	78 ± 6	60 ± 4
2,3,5-TCP	87 ± 4	70 ± 5	44 ± 4
2,3,6,TCP	83 ± 2	75 ± 2	77 ± 5
2,4,5-TCP	75 ± 4	67 ± 5	66 ± 5
2,4,6-TCP	88 ± 5	70 ± 6	66 ± 3
3,4,5-TCP	5	ND[b]	ND
	Potting Soil		
2,4,5-TCP	81 ± 2	62 ± 8	51 ± 3

[a] Mean ± SD (n = 4).
[b] ND = not detected.

Table 2. Recovery (%) of substituted phenols from clay soil containing 100 μg/50 g of each phenol [a]

Phenol	Nielsen–Kryger procedure	Exhaustive distillation procedure
Phenol	44 ± 8	56 ± 7
2,4-Dimethylphenol	42 ± 1	99 ± 2
4-Chlorocresol	35 ± 3	96 ± 3
2-Chlorophenol	59 ± 4	97 ± 2
2,4-Dichlorophenol	64 ± 4	92 ± 2
2,4,6-TCP	70 ± 2	98 ± 1
2-Nitrophenol	69 ± 4	91 ± 2
4-Nitrophenol	ND[b]	4 ± 1
2,4-Dinitrophenol	40 ± 5	24 ± 3
4,6-Dinitrophenol	25 ± 7	43 ± 7
PCP	77 ± 4	95 ± 1

[a] Mean ± SD (n = 4).
[b] ND = not detected.

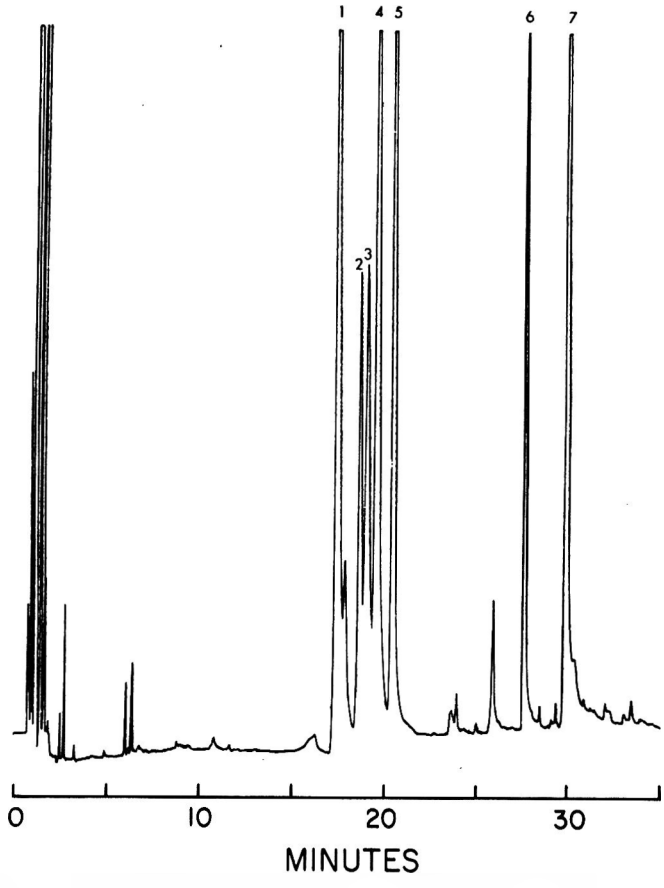

Figure 1. Gas chromatogram of chlorinated phenols on fused silica SE 54 column with electron capture detection. Peak identities: (1) 2,3,5-TCP; (2) 2,4,6-TCP; (3) 2,4,5-TCP; (4) 2,3,4-TCP; (5) 2,3,6-TCP; (6) 3,4,5-TCP; (7) PCP.

had mixed results. 2-Chloro- and 2,4-dichlorophenols, as expected, were isolated in satisfactory yield; but of 4 nitrophenols, only 2-nitrophenol was isolated in moderate yield. Recoveries of 2,4- and 4,6-dinitrophenol were low, and 4-nitrophenol could not be recovered. This is a classic case of intramolecular vs intermolecular hydrogen bonding (12). 2-Nitrophenol, which forms intramolecular hydrogen bonds, has a lower boiling point and is less soluble in water; it is therefore steam-volatile and could be isolated in satisfactory yield. 4-Nitrophenol, with its intermolecular hydrogen bonds, has a very high boiling point and greater solubility in water; these characteristics inhibit its steam distillation. Perhaps the same holds true for 3,4,5-TCP which could not be recovered satisfactorily.

Recoveries of phenol (which is steam-volatile), 2,4-dimethylphenol, and 4-chlorocresol were also very low. As mentioned earlier, this could be due to the inefficiency of the extracting solvent. To assess this, a 50 g sample spiked with substituted phenols was placed in a 3-neck round-bottom flask and, after treatment in a similar manner as described before, was steam-distilled. No organic solvent was placed in the distillation column. The distillate was collected via a short glass tube into a 125 mL filtration flask containing 30 mL methylene chloride and was stirred with a magnetic stirrer. The excess water was allowed to return continuously to the distillation flask via short glass tubing. At the end of 2 h, the organic layer was separated, dried over anhydrous sodium sulfate, and concentrated in a Kuderna–Danish apparatus. The methylene chloride was carefully displaced with hexane, and the extract was analyzed on the 1240DA column with a flame ionization detector. The results are shown in Table 2. The recoveries of these phenols by the exhaustive distillation procedure are much higher than those obtained by the Nielsen-Kryger technique. This clearly

shows that the efficiency of the extracting solvent is a major factor affecting recoveries of these phenols from soil.

The Nielsen-Kryger steam distillation procedure appears to offer a simple, convenient method for isolation of chlorinated phenols from soil. Recoveries compare favorably with those by Soxhlet extraction, shaking with organic solvent, or sonication. The method requires only a small amount of solvent for extraction, thus eliminating losses due to volatilization, which are uncontrollable with a rotary evaporator or a Kuderna-Danish concentrator. It also eliminates the extensive cleanup step to remove numerous undesirable artifacts which other extraction techniques leave in the raw extract. The only impurity that interferes with the analysis is sulfurous material which is co-distilled during steam distillation. This impurity can be conveniently removed by shaking with a few drops of metallic mercury (13).

One advantage of using a fused silica or 1240DA column for analysis is that derivatization of the phenols is unnecessary (Figure 1).

The method has more limited utility for recovery of other substituted phenols which are highly soluble in water. However, in view of recent reports suggesting that similar techniques permit the efficient recovery of a variety of nonphenolic organic compounds, the method described here may find application as a rapid screening technique for a variety of contaminants in soil.

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Interlaboratory Study of the Hall 700A Halogen Electrolytic Conductivity Gas Chromatographic Detector

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An interlaboratory study of the Hall 700A (halogen) electrolytic conductivity GC detector was carried out in 8 Food and Drug Administration laboratories. The study sought to provide data to support the use of the Hall 700A (halogen) detector as an alternative to the electron capture and microcoulometer detectors in the determination of pesticide and industrial chemical residues. The following characteristics of the detector were studied: linear dynamic range for 12 organohalogen pesticides and industrial chemicals injected over a 0.05-27.00 μg/mL range; repeatability of response; quantitation of 5 pesticides and Aroclor 1254 added to food extracts (fish and green peppers) cleaned up by commonly used multiresidue analytical methods; selectivity, defined as response to a halogenated compound (chlorpyrifos) vs response to a nonhalogenated compound (caffeine). The interlaboratory study showed that halogen response and selectivity are independent factors which define the performance of the Hall 700A (halogen) detector. Recommendations are provided to ensure consistent and selective halogen response. Finally, the study provides adequate data to support continued use of the Hall 700A (halogen) detector in the determination of pesticides and industrial chemicals.

Gas chromatography (GC) employs various types of detectors, depending on the particular analyte or class of compounds being analyzed. Selective detectors are more useful than universal detectors because they preferentially respond to analytes containing certain moieties or atoms and consequently provide additional information about the identity of the analyte.

Coulson (1) developed a GC detector to determine halogen- or nitrogen-containing compounds by measuring the change in conductivity resulting from the ionization of these compounds. Although this detector operated on a unique or selective basis, it was found in practice to lack sensitivity and to be very difficult to maintain at optimum conditions. Hall (2) modified this detector system, solving some of the mechanical problems as well as improving the sensitivity. Although this microelectrolytic conductivity system offered better sensitivity, practical application in normal pesticide residue analysis was difficult because of frequent adsorption of analytes in the transfer line, poor catalytic conversion of nitrogen-containing compounds with extended use, and need for frequent maintenance to achieve optimum conditions.

In 1976, Hall (3) further refined this microelectrolytic conductivity detector system. In the new system, the quartz pyrolysis tube was replaced with a nickel tube giving better stability, and the detector was further miniaturized to allow installation onto a gas chromatograph, which eliminated the need for a transfer line from the GC column to the reactor. This detector, called the Hall 700A, offers selective determination of organic compounds containing halogen, nitrogen, and sulfur. Since its introduction, it has enjoyed wide acceptance.

The Hall 700A detector, manufactured by Tracor Inc., Austin, TX, consists of a differential conductivity cell, reactor, signal processing module, and solvent reservoir module. In the halogen mode, a stream of *n*-propanol (termed solvent) enters the differential conductivity cell. The solvent stream is pumped at a specific rate through the reference electrode (top conductivity cell). The solvent flows through the conductivity cell into the gas-liquid contactor. Here the GC effluent, exiting from the reactor with its halogenated constituents now reduced in the presence of hydrogen gas to HX, bubbles into the solvent where HX ionizes and changes the conductivity of the solvent. This gas-liquid mixture flows into the separator and the gas phase is removed from the solvent. The solvent then passes through the analytical electrode (bottom conductivity cell). The gas phase and solvent stream remix at the exit of the conductivity cell. This mixture flows into the solvent reservoir module to be cleaned up by an ion exchange resin column. The clean solvent then recirculates through the conductivity cell. In the halogen mode, *n*-propanol is used so that only strong acids, e.g., HCl, will ionize in the solvent stream. Interferences from other acidic compounds formed in the reactor are effectively eliminated since these products will not ionize and change the conductivity of the *n*-propanol solvent (4). With the increased use by FDA of the Hall 700A detector both in conjunction with and in place of electron capture, microcoulome-

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Table 1. Composition of samples used in study of Hall 700A GC detector

Vial	Constituents
	Phase 1
1	pentachloroanisole 0.05 μg/mL; chlorpyrifos, dieldrin, captan, *cis*-nonachlor, *p,p'*-DDT, each 0.10 μg/mL
2	pentachloroanisole 0.25 μg/mL; chlorpyrifos, dieldrin, captan, *cis*-nonachlor, *p,p'*-DDT, each 0.50 μg/mL
3	pentachloroanisole 0.50 μg/mL; chlorpyrifos, dieldrin, captan, *cis*-nonachlor, *p,p'*-DDT, each 1.00 μg/mL
4	pentachloroanisole 2.50 μg/mL; chlorpyrifos, dieldrin, captan, *cis*-nonachlor, *p,p'*-DDT, each 5.00 μg/mL
5	pentachloroanisole 5.00 μg/mL; chlorpyrifos, dieldrin, captan, *cis*-nonachlor, *p,p'*-DDT, each 10.00 μg/mL
6	di-isobutyl phthalate 100 ng; ethion 100 ng; chlorpyrifos 1 ng; methyl palmitate 100 ng; caffeine 100 ng; octadecane 2 μg[a]
	Phase 2
7	β-BHC, 2-chloronitrobenzene, 2,3-dichloronitrobenzene, 2,3,4-trichloronitrobenzene, each 0.10 μg/mL; 4-chlorobiphenyl 0.20 μg/mL; 4-bromobiphenyl 0.27 μg/mL
8	β-BHC, 2-chloronitrobenzene, 2,3-dichloronitrobenzene, 2,3,4-trlchloronitrobenzene, each 0.50 μg/mL; 4-chlorobiphenyl 1.00 μg/mL; 4-bromobiphenyl 1.35 μg/mL
9	β-BHC, 2-chloronitrobenzene, 2,3-dichloronitrobenzene, 2,3,4-trlchloronitrobenzene, each 1.00 μg/mL; 4-chlorobiphenyl 2.00 μg/mL; 4-bromobiphenyl 2.70 μg/mL
10	β-BHC, 2-chloronitrobenzene, 2,3-dichloronitrobenzene, 2,3,4-trichloronitrobenzene, each 5.00 μg/mL; 4-chlorobiphenyl 10.00 μg/mL; 4-bromobiphenyl 13.50 μg/mL
11	β-BHC, 2-chloronitrobenzene, 2,3-dichloronitrobenzene, 2,3,4-trichloronitrobenzene, each 10.00 μg/mL; 4-chlorobiphenyl 20.00 μg/mL; 4-bromobiphenyl 27.00 μg/mL
	Phase 3
12	Aroclor 1254, 6.80 μg/mL
13	6% eluate fish (Method A) fortified at 1.0 ppm with Aroclor 1254
14	Aroclor 1254, 6.80 μg/mL
15	dieldrin 0.662 μg/mL; endosulfan sulfate 1.37 μg/mL
16	15% eluate fish (Method A) fortified at 0.10 ppm with dieldrin
17	dieldrin 0.662 μg/mL; endosulfan sulfate 1.37 μg/mL
18	50% eluate fish (Method A) fortified at 0.20 ppm with endosulfan sulfate
19	dieldrin 0.662 μg/mL; endosulfan sulfate 1.37 μg/mL
20	di-isobutyl phthalate 100 ng; ethion 100 ng; chlorpyrifos 1 ng; methyl palmitate 100 ng; caffeine 100 ng; octadecane 2 μg[a]
	Phase 4
21	lindane 0.262 μg/mL; DCPA 0.485 μg/mL; *p,p'*-DDE 0.510 μg/mL
22	6% eluate peppers (Method B) fortified at 0.05 ppm with lindane and 0.10 ppm with *p,p'*-DDE
23	lindane 0.262 μg/mL; DCPA 0.485 μg/mL; *p,p'*-DDE 0.510 μg/mL
24	di-isobutyl phthalate 100 ng; ethion 100 ng; chlorpyrifos 1 ng; methyl palmitate 100 ng; caffeine 100 ng; octadecane 2 μg[a]
25	15% eluate peppers (Method B) fortified at 0.10 ppm with DCPA
26	lindane 0.262 μg/mL; DCPA 0.485 μg/mL; *p,p'*-DDE 0.510 μg/mL
27	di-isobutyl phthalate 100 ng; ethion 100 ng; chlorpyrifos 1 ng; methyl palmitate 100 ng; caffeine 100 ng; octadecane 2 μg[a]
28	Method C peppers fortified at 0.05 ppm with lindane, 0.10 ppm with *p,p'*-DDE, and 0.10 ppm with DCPA
29	lindane 0.262 μg/mL; DCPA 0.485 μg/mL; *p,p'*-DDE 0.510 μg/mL
30	di-isobutyl phthalate 100 ng; ethion 100 ng; chlorpyrifos 1 ng; methyl palmitate 100 ng; caffeine 100 ng; octadecane 2 μg[a]
30A	lindane 0.130 μg/mL; DCPA 0.291 μg/mL; *p,p'*-DDE 0.306 μg/mL
31	Method D peppers fortified at 0.05 ppm with lindane, 0.10 ppm with *p,p'*-DDE, and 0.10 ppm with DCPA
32	lindane 0.130 μg/mL; DCPA 0.291 μg/mL; *p,p'*-DDE 0.306 μg/mL
33	di-isobutyl phthalate 100 ng; ethion 100 ng; chlorpyrifos 1 ng; methyl palmitate 100 ng; caffeine 100 ng; octadecane 2 μg[a]

[a] Amount injected.

tric, and thermionic detectors in the determination of residues through AOAC multiresidue methods, detector performance became an important issue. The Hall 700A (halogen) detector was first evaluated by a single laboratory (5). This evaluation generated data to support proposed general operating parameters for determining pesticides and industrial chemicals. The proposed general operating parameters were then tested among 8 Food and Drug Administration (FDA) laboratories. The data from this interlaboratory study are presented here. The study focused on selectivity (halogenated vs nonhalogenated responses), repeatability of re-

sponse, quantitation of residues (pesticides and industrial chemicals), and linear dynamic range of the Hall 700A (halogen) detector.

Experimental

Each laboratory was instructed to proceed as follows:

(1) Prepare a 6 ft × 2 mm id GC column of 5% OV-101 (or equivalent) on 80–100 mesh Chromosorb WHP. Condition GC column by increasing temperature 5°C/min from ambient to 230°C. Hold at maximum temperature for 8 h minimum. Use a GC column flow rate of ca 30 mL/min. Operate the column at 200°C, except in Phase 2 operate at 130°C.

(2) Adjust column temperature, if necessary, to give retention time relative to chlorpyrifos (RR_c) for *p,p'*-DDT = 3.10.

(3) Operate Hall 700A detector under the following conditions: (a) solvent flow rate, 0.35 mL/min; (b) hydrogen flow rate, 60–100 mL/min; (c) electrometer range, 10 in OPR/FLT mode; (d) carrier gas helium, 30–60 mL/min; (e) injection port temperature 210°C; (f) reactor tube temperature 800°C minimum; (g) reactor base temperature 250°C.

(4) Prepare appropriate concentrations of standard solutions: chlorpyrifos, pentachlorobenzene, and mixture of lindane, heptachlor, chlorpyrifos, heptachlor epoxide, dieldrin, endrin, and *p,p'*-DDT in isooctane (2,2,4-trimethylpentane).

(5) Adjust electrometer attenuator so that 1.5 ng chlorpyrifos at 200°C GC column temperature and 1.5 ng pentachlorobenzene at 130°C GC column temperature give 30–70% full scale deflection (FSD).

The study was divided into 4 phases. Each phase was to be completed in one day. Phases 1 and 2 examined linear dynamic range of 12 organohalogen compounds over a concentration range of 0.05–27.00 μg/mL (Table 1). Phase 1 tested compounds which normally elute from a GC column at 200°C, such as dieldrin; Phase 2 tested compounds with lower boiling points, including a brominated compound, which normally elute from a GC column at 130°C, such as 2-chloronitrobenzene. Phases 3 and 4 examined the ability of the Hall 700A detector to quantitatively determine pesticides and industrial chemicals at normal residue levels in cleaned-up extracts of fatty and nonfatty foods, respectively. Samples prepared by the following 4 multiresidue methods were examined:

Method A: secs **29.012, 29.014,** and **29.015,** *Official Methods of Analysis* (6).

Method B: secs **29.011** and **29.015** (6).

Method C: Described by Carson (7).

Method D: Described by Luke et al. (8).

Each participant received 34 injection vials (Table 1) containing solutions prepared in the author's laboratory. All standard solutions, stock and dilutions, were prepared in pesticide grade isooctane. Before fortification, each cleaned-up sample extract was examined by a Hall 700A (halogen) detector to verify that the extracts did not contain residues of the compounds to be added or other interferences. Sample solutions for Phases 3 and 4 were fortified after analysis to eliminate the possible variation in recovery through each method. Therefore, the amount of added compound determined reflects the detector performance only.

Each participant was instructed to weigh the injection vials for each phase before starting that phase. If the weight varied more than ±0.025 g from the weight stated for that vial, a replacement was provided. Each participant was instructed to inject 3 μL from each vial by solvent flush or difference technique. The participants monitored their GC system by injecting a mixed pesticide standard solution before and after each phase.

Each participant clearly labeled all chromatograms and submitted them without measuring peak size. The author measured all peak heights and performed the calculations required for interpretation of the data. This ensured consistent quantitation of all responses.

Results and Discussion

Linear Dynamic Range

Phase 1 examined the linear dynamic range, i.e., range of measurable response for increasing amounts of injected material, of the Hall 700A detector for 6 organochlorine pesticides. Two injections per vial were made in consecutive order for vials 1–6. The average response for each compound was used to calculate its response factor. Figure 1 shows the linear dynamic range for chloropyrifos obtained by the 8 participating laboratories. (Note: Laboratory 4 was not able to participate after receipt of vials and instructions.) The linear dynamic range graph is generated by plotting response factor vs log of concentration. Response factor is the average response (peak height) times attenuation divided by the amount of compound injected. An ideal linear dynamic range would produce a horizontal line over a 10^4 or greater range of concentration. In Figure 1, Laboratories 1, 5, and 6

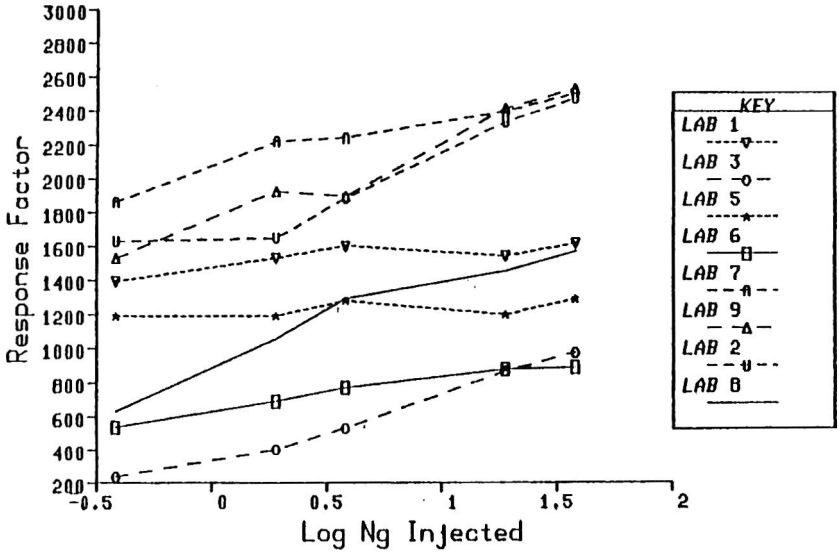

Figure 1. Linear dynamic range plot of chlorpyrifos.

exhibit the greatest linear dynamic range for chlorpyrifos.

Figure 2 shows similar graphical representation for captan and *p,p'*-DDT by all 8 laboratories. In Phase 1, captan exhibited the poorest response in all laboratories. The poor response to captan is due not to the inability of the Hall 700A detector to determine this compound, but more likely to GC column effects, including adsorption. Two detectors gave no response to captan at the lowest concentration. The linear dynamic range for *p,p'*-DDT is representative of the other Phase 1 compounds (see Table 1) for all laboratories. The detectors in all laboratories had similar linear dynamic ranges for these compounds. Vial 6, selectivity solution, will be discussed later.

Phase 2, vials 7–11, monitored the linear dynamic range of β-BHC and 5 industrial chemicals. Two injections per vial were made in consecutive order. Figure 3 depicts this range for 4-bromobiphenyl and 2,3-dichloronitrobenzene. The lowest absolute response for all compounds in Phases 1 and 2 was obtained for 4-bromobiphenyl. Laboratories 3, 6, 8, and 9 had no detectable response at the lowest concentration injected (0.27 μg/mL). Consequently, the greatest linear dynamic range for any laboratory was only a 10^2 concentration range. The linear dynamic range for 2,3-dichloronitrobenzene is representative of the other industrial chemicals in Phase 2 (see Table 1) and exhibits linear response for detectors in all 8 laboratories.

The response data for Laboratory 2 for Phases 1 and 2 were divided by a constant, 4, so that the data for all laboratories could be presented on a single graph. There is no apparent reason why the detector used by Laboratory 2, operated at the same parameters as the other 7 detectors, exhibited this large absolute response. Although Laboratory 2 response data represent the most extreme case, the response data for the other 7 laboratories span a wide range for every concentration. Figure 1 illustrates this point where the response factor for chlorpyrifos from the 7 laboratories varies 200–1900 at the lowest concentration. This difference in absolute response among all participants indicates that the parameters prescribed do not assure consistent response among laboratories. Even though this variation in absolute response existed, it did not affect the quantitative determination of pesticides and industrial chemicals in Phases 3 and 4.

Determination of Compounds Added to Food Extracts

Phase 3 examined the ability of the Hall 700A detector to determine residues in a fatty food (fish): Method A. Injections of standard solutions alternated with injection of sample solutions (see Table 1) fortified with 1.0 ppm Aroclor 1254, 0.1 ppm dieldrin, and 0.2 ppm endosulfan sulfate. The standard solution gave a response within ±10% of the response of the sample residue. Peak height measurements were used for

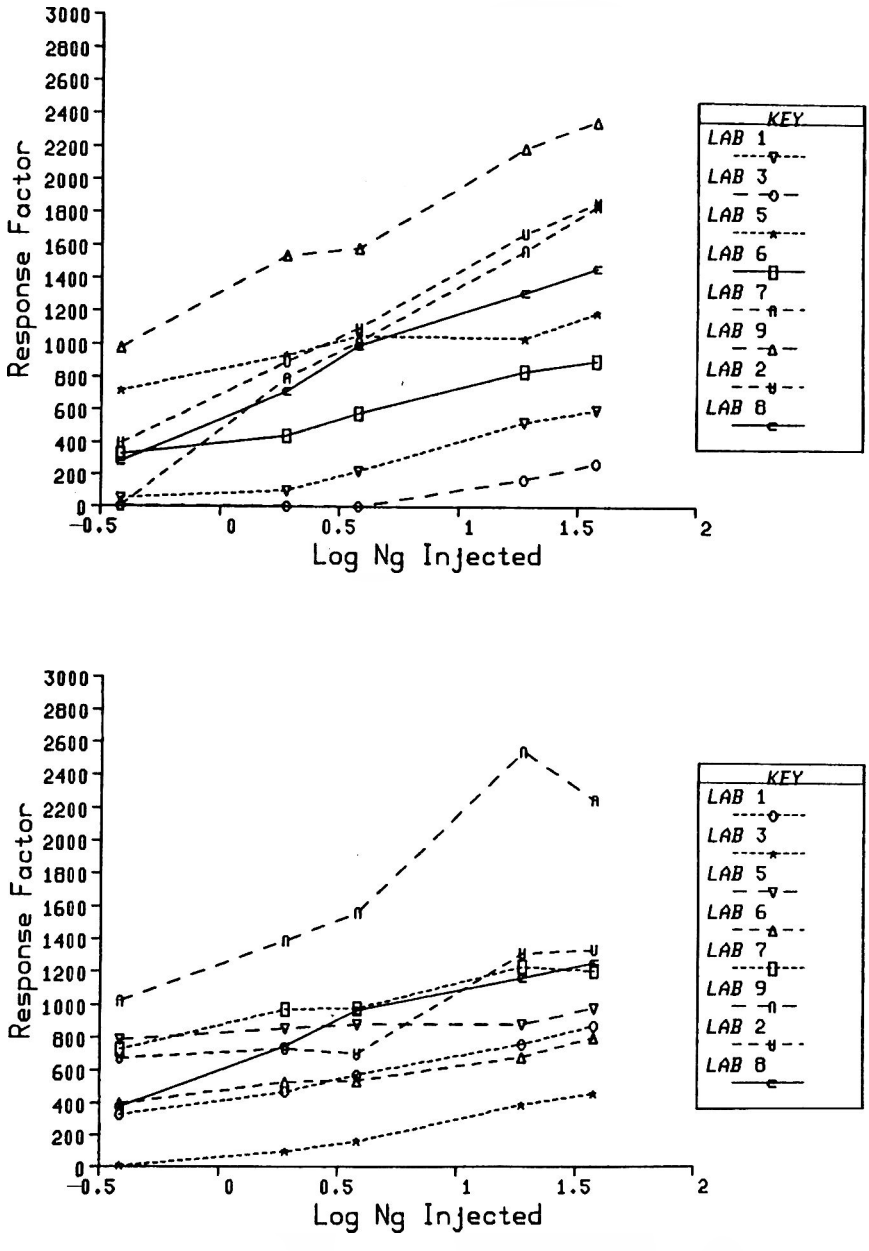

Figure 2. Linear dynamic range plots of captan and *p,p'*-DDT.

quantitation because neither the sample nor the standard response peak shape exhibited broadening. The average peak height of the standard solution injections was used for quantitation.

A summary of the results for Phase 3 is given in Table 2. In all cases, the mean results for Aroclor 1254, dieldrin, and endosulfan sulfate exceeded 100% (104–113%). This could not be attributed to injection error alone, since the maximum variability for a given compound within any laboratory was less than 7%. The equivalent of 19 mg (equal to 0.36 mg fat basis) sample material was injected from vials 13, 16, and 18.

Phase 4 examined the ability of the Hall 700A (halogen) detector to determine residues in green pepper extracts: Methods B, C, and D. The sample extracts were fortified with 0.05 ppm lindane, 0.1 ppm DCPA (Dacthal), and 0.1 ppm *p,p'*-DDE. Standard and sample solutions were

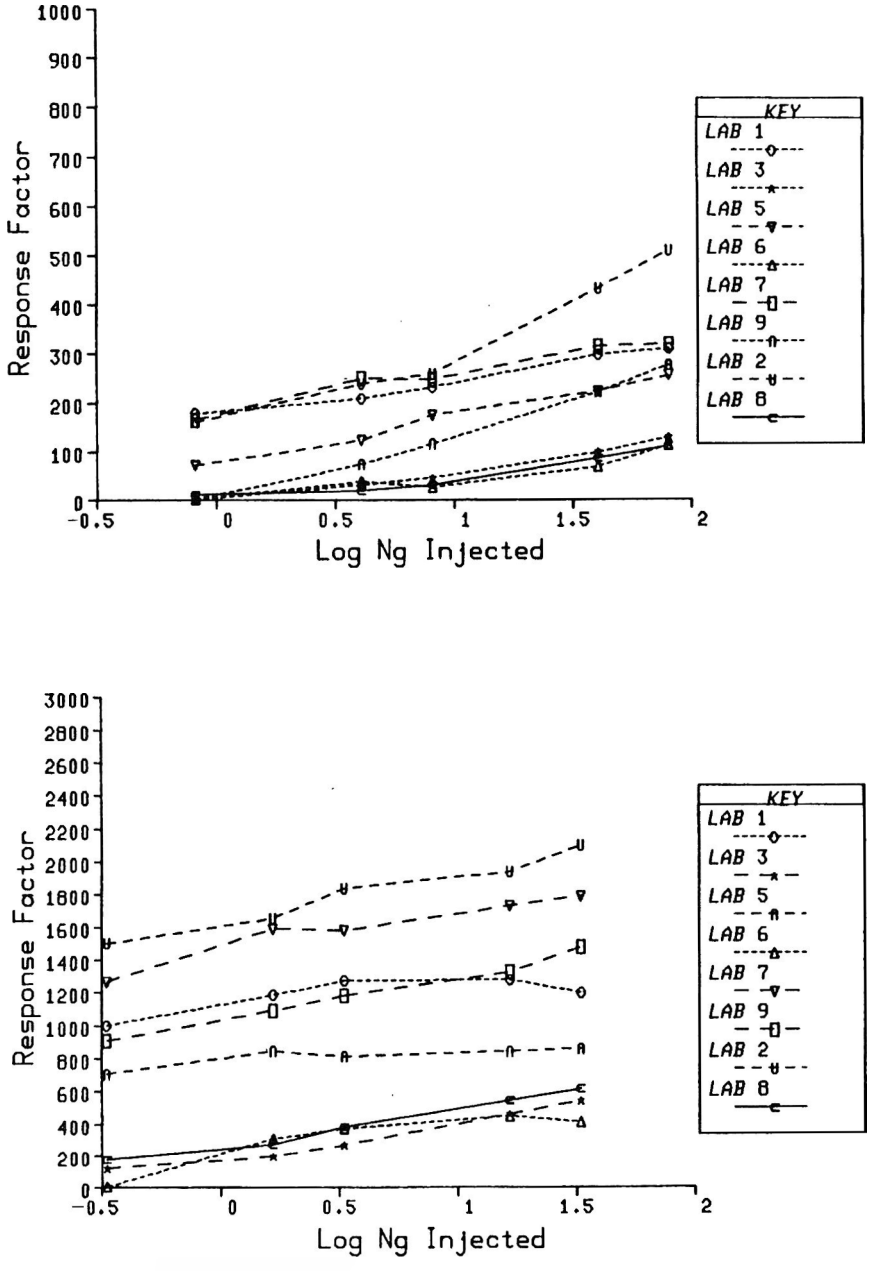

Figure 3. Linear dynamic range plots of 4-bromobiphenyl and 2,3-dichloronitrobenzene.

injected alternately (see Table 1). The average peak height responses for the solutions for each laboratory were used to calculate the level of added compound present in the sample solutions. Standard and sample response were matched closely, as in Phase 3, so that peak height measurements could be used for quantitation.

Results for all added compounds by Method B exceeded 100% but the determinations were in good agreement among all laboratories (coefficient of variation (CV) 5–7%). It was anticipated that residue levels determined for cleaned-up extracts from Methods A and B, which are AOAC official methods, would not exceed a range of 90 to 110% (9). Given the same method for quan-

Table 2. Summary of results of added compound determinations obtained by using Hall 700A (halogen) detector

Pesticide	Recovery, % of added pesticide	Mean, %	CV, %
Method A: Fish Extract			
Aroclor 1254	105–123	113	5
Dieldrin	97–119	110	6
Endosulfan SO_4	95–112	104	6
Method B: Green Pepper Extract			
Lindane	101–118	111	5
DCPA	103–132	116	7
p,p'-DDE	104–120	110	5
Method C: Green Pepper Extract			
Lindane	71–103	91	12
DCPA	80–122	99	13
p,p'-DDE	88–110	101	8
Method D: Green Pepper Extract			
Lindane	79–100	87	7
DCPA	91–122	103	10
p,p'-DDE	97–114	106	6

titation and elimination of possible bias due to method of quantitation, the data are slightly high in this study. However, the residue determinations made with the Hall 700A were reasonable and within experimental error.

The results for lindane and DCPA by Method C exhibit the largest variation, as indicated by CV (Table 2). Laboratories 1, 3, and 8 reported the lowest percent of added compound determined (71–88%). The Method C final solution may contain traces of acetonitrile from the charcoal cleanup column eluant. The acetonitrile, which contains nitrogen, may have affected the responses for halogenated compounds observed in these laboratories. This point is discussed further under *Selectivity*.

Injection repeatability for lindane, DCPA, and *p,p'*-DDE, calculated from response (peak height) data for injections of vials 21, 23, 26, and 29, is shown in Table 3. The CV range is much greater, 0.3–18%, than that of Phase 3. Note that Laboratories 3, 6, and 8 exhibit the greatest variation (CV $\geq$ 5%). In Phase 4 for Method B and Method C solutions, sample size injected was equivalent to 18 mg.

For Method D, the sample size injected was equivalent to 9 mg. Fortification of the final solution was made at the same ppm level as above (see Table 1). A summary of the results for all 8 laboratories is given in Table 2.

In contrast to Method C, Method D final solution includes no nitrogen-containing solvent, yet lindane has a mean for all laboratories of 87%. This low percent of added compound determined also has a relatively good CV of 7%. The low level determined and good reproducibility

Table 3. Phase 4 study: injection repeatability of Hall 700A detector [a]

Lab.	Compd	Max. [b]	Min. [b]	Mean [b]	SD	CV, %	Range
1	lindane	163.0	149.0	158.8	6.5	4.1	14.0
	DCPA	119.0	108.0	114.0	4.7	4.1	11.0
	p,p'-DDE	74.5	74.0	74.1	0.3	0.3	0.5
2	lindane	180.0	153.5	170.9	11.9	7.0	26.5
	DCPA	65.5	58.5	63.0	3.1	4.9	7.0
	p,p'-DDE	105.0	93.5	100.8	5.0	5.0	11.5
3	lindane	152.0	123.5	139.9	14.4	10.3	28.5
	DCPA	136.0	121.0	128.8	8.1	6.3	15.0
	p,p'-DDE	94.0	81.0	87.4	6.6	7.5	13.0
5	lindane	128.0	121.0	123.3	3.3	2.7	7.0
	DCPA	93.0	87.5	90.9	2.7	2.9	5.5
	p,p'-DDE	65.5	60.0	62.8	2.9	4.6	5.5
6	lindane	98.0	67.8	83.6	14.3	17.1	30.2
	DCPA	71.5	49.8	62.0	10.7	17.3	21.7
	p,p'-DDE	48.5	33.4	40.3	7.4	18.3	15.1
7	lindane	135.5	113.0	124.1	9.3	7.5	22.5
	DCPA	105.0	90.0	94.9	7.0	7.4	15.0
	p,p'-DDE	68.0	64.0	65.3	1.8	2.8	4.0
8	lindane	137.0	110.0	125.1	13.3	10.6	27.0
	DCPA	112.5	93.0	103.6	9.8	9.5	19.5
	p,p'-DDE	82.5	70.0	76.6	6.5	8.5	12.5
9	lindane	153.0	149.0	150.6	1.8	1.2	4.0
	DCPA	132.5	129.5	131.0	1.5	1.1	3.0
	p,p'-DDE	100.5	95.5	98.0	2.0	2.1	5.0

[a] Four observations were made for each compound.
[b] Peak height response in mm units.

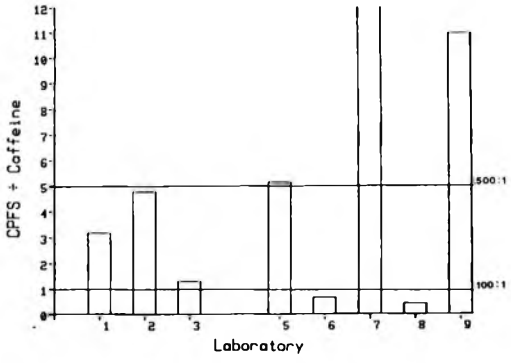

Figure 4. Selectivity ratio, chlorpyrifos:caffeine, for all laboratories.

between laboratories indicate that lindane in Method D extract is more difficult to determine than DCPA and *p,p'*-DDE with the Hall 700A detector.

A mixed standard solution which contained lindane, heptachlor, chlorpyrifos, heptachlor epoxide, dieldrin, endrin, and *p,p'*-DDT was injected at the start and completion of each phase. This mixed standard solution provided a benchmark to monitor detector response stability. After the injection of Method D sample extract, the *p,p'*-DDT standard in the mixed standard chromatogram was degraded to some extent by all 8 GC systems. The *p,p'*-DDT peak height response decreased 20–70% and a peak appeared at the retention time of *p,p'*-TDE, 2.49 RR_c. The participants noted that this phenomenon was temporary, and after several hours the *p,p'*-DDT response returned to normal with a corresponding disappearance of the "apparent" *p,p'*-TDE peak.

Selectivity

Throughout the study, a selectivity solution was injected to test the ability of the Hall 700A detector to preferentially determine organohalogen compounds. Injections were made in Phase 1 (vial 6), Phase 3 (vial 20), and Phase 4 (vials 24, 27, 30, and 33). The selectivity solution contained organic compounds with different chemical moieties and elements, namely: methyl palmitate (fatty acid ester), di-isobutyl phthalate (phthalic acid ester), ethion (P, S), chlorpyrifos (Cl, P, N, S), caffeine (N), and octadecane (C). Selectivity solution, Table 1, is listed as amount injected. Caffeine was the only nonhalogenated compound that gave a response, except for Laboratory 7 whose detector gave no response to caffeine.

The selectivity of the Hall 700A detector in the halogen mode may be measured as a ratio of the response to a halogenated compound vs the response to a nonhalogenated compound. Since caffeine was the only nonhalogenated compound to produce a response in this study, the selectivity ratio was defined as (response (mm)/amount (ng)) chlorpyrifos:(response (mm)/amount (ng)) caffeine. Figure 4 presents the selectivity ratio of the Hall 700A detector calculated for each laboratory. The *y*-axis value is the average chlorpyrifos:caffeine response ratio of the 6 injections of the selectivity solutions made throughout the study. An equal response for both chlorpyrifos and caffeine would yield a value of 1 on the *y*-axis. At this level of response for chlorpyrifos and caffeine, the corresponding selectivity ratio is 100:1, as 1 ng chlorpyrifos and 100 ng caffeine were injected each time. (Note: chlorine/chlorpyrifos (Mw/Mw) = 0.29 and nitrogen/caffeine (Mw/Mw) = 0.32). As the response to nonhalogenated compound approaches zero, the selectivity ratio becomes exceedingly large. The selectivity ratio for chlorpyrifos vs all other compounds in the selectivity solution is $\gg$1000:1. Figure 5 shows a chromatogram of vial 6, selectivity solution, for Laboratory 1.

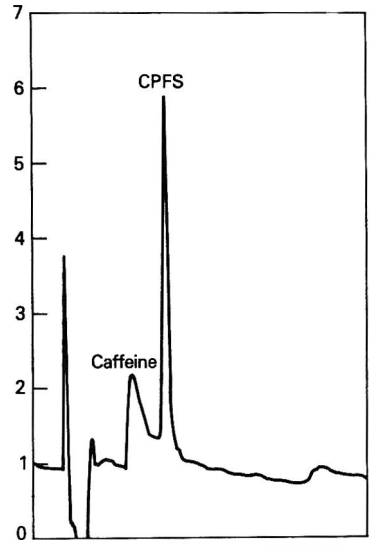

Figure 5. Chromatogram of 3 μL injections of vial 6, selectivity solution, by Laboratory 1. Selectivity ratio of 300:1.

Laboratories 1, 3, 6, and 8 have selectivity ratios $\leq$500:1, all other laboratories exceed this ratio. The laboratories having a poor selectivity ratio, $\leq$500:1, will have a greater tendency to detect nonhalogenated compounds, especially nitro-

gen-containing compounds as shown in this study. This greater response to nitrogen-containing compounds may lead to practical limitations because of effects of co-extractives and solvents containing nitrogen. For example, Method C extract may contain traces of acetonitrile. This nitrogen-containing compound may have caused the tailing baseline evident in the chromatograms from laboratories exhibiting ≤500:1 selectivity ratio. The relatively large CV (8–13%) reflects some difficulty in determining lindane and DCPA by these 4 laboratories.

Information available on the Hall 700A detector suggests that several factors affect the selectivity: condition of the resin, solvent, reactor tube, and reactor tube temperature. The most important factor is certainly the resin bed condition. The resin bed has a limited capacity to remove ionic species from the solvent and maintain the slightly acidic solvent medium. As the capacity of the resin is exceeded, the solvent becomes a better ionizing medium and other reductive products may ionize. The resin bed integrity may be assured by monitoring the selectivity ratio. When the selectivity ratio falls below 500:1, the resin bed should be repacked and the reservoir solvent should be replaced. For example, Laboratory 8, which exhibited a selectivity ratio of 30:1, improved its selectivity to 700:1 merely by replacing the resin bed and solvent.

Reactor tube and reactor tube temperature affect selectivity to a lesser extent. Reactor tubes exhibit variable halogen response when compared to one another on the same system. We have no data on which to predict the relative halogen response that a particular reactor tube will exhibit. But if selectivity is still low (≤500:1) after replacement of resin and solvent, then changing the reactor tube may improve the halogen response and consequently the selectivity.

Reactor tube temperature must be kept high enough to ensure complete reduction of halogenated compounds. Operating the reactor tube temperature at a minimum setting of 900°C should suffice. (Note: Maximum temperature setting is 1000°C.) In addition, purity of the gases, both helium and hydrogen, has been shown to be of primary importance to the operation of the Hall 700A detector. One laboratory, Laboratory 1, was unable to obtain high quality helium and successfully substituted hydrogen as the carrier gas.

Poor selectivity is not necessarily evident in the chromatograms of organohalogen compounds. For example, Laboratory 8 exhibited poor selectivity (30:1). However, the GC system still performed adequately to give a typical chromatogram for organochlorine pesticides, as shown in Figure 6. The analyst must be cognizant that selectivity and efficiency of the GC system do not necessarily coincide. In maintaining the Hall 700A detector/GC system, the analyst must monitor halogen response, selectivity, and GC peak shape separately and routinely.

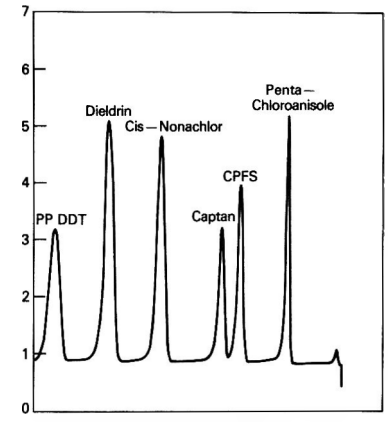

Figure 6. Chromatogram of 3 μL injection of vial 3 by Laboratory 8.

Conclusions

Evaluation of the data from this study clearly shows that halogen response, peak shape, and selectivity are independent variables affecting the utilization of the Hall 700A detector/GC system. This study of the Hall 700A detector has provided data to support several recommendations for its operation. The following parameters differ from those recommended by Tracor Instruments (4, 10):

(1) Use ultra-high purity (99.995%) helium and hydrogen gases.

(2) Maintain hydrogen flow rate at 60–100 mL/min (5).

(3) Operate reaction tube temperature at 900°C setting minimum.

(4) Maintain solvent flow rate at about 0.35 mL/min (5).

(5) Monitor selectivity ratio, chlorpyrifos: caffeine, and maintain selectivity ratio of 500:1 or better.

(6) Monitor halogen response, using a mixed standard solution containing at least lindane, chlorpyrifos, and *p,p'*-DDT.

In this study the Hall 700A (halogen) detector

has been shown to adequately determine pesticides and industrial chemicals in extracts of foods prepared by commonly used analytical methods. This selective and sensitive detector may be used as an alternative to electron capture and microcoulometric detectors in conjunction with multiresidue analytical methodology.

Acknowledgments

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Identification of Chlorinated Nitrobenzene Residues in Mississippi River Fish

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Residues of lower chlorinated nitrobenzenes have been found at levels up to about 1 ppm in 8 samples of Mississippi River fish. Electron capture gas chromatography (EC/GC) was used for determination after extraction and cleanup using a procedure based on the AOAC multiresidue method for organochlorine and organophosphorus pesticides in nonfatty foods. The residues found included 2-, 3-, and 4-chloronitrobenzene and 2,3- and 3,4-dichloronitrobenzene; identity was confirmed by GC/mass spectrometry. GC retention times for 15 monochloro- through pentachloro-substituted nitrobenzene congeners were determined with OV-101 and mixed OV-101 + OV-210 columns at 130°C. In studies of the nonfatty food extraction and cleanup procedures of the AOAC method, recoveries of 15 chlorinated nitrobenzenes from spiked fish samples ranged from 68 to 116%. GC of cleaned up fish extract aliquots equivalent to 20 mg sample allowed quantitation of individual congeners at levels of about 0.025 and 0.005 ppm with ^{3}H and ^{63}Ni EC detectors, respectively. The contamination of Mississippi River fish with chlorinated nitrobenzenes appears to be localized in a 150 mile section of the river extending from St. Louis, MO, to Cape Girardeau, MO; no chlorinated nitrobenzenes (<0.005 ppm) were detected in Mississippi River fish caught above or below this region of the river or in fish from the lower Missouri River, which joins the Mississippi River near St. Louis.

Food and Drug Administration (FDA) personnel use the AOAC official multiresidue method for organochlorine and organophosphorus pesticides (secs. **29.001-29.018** (1)) to analyze foods for many potentially hazardous contaminants besides those for which the method has official status (2). Since 1976, FDA monitoring programs for pesticide and industrial chemical residues in foods have included analyses of selected food samples, mainly of domestic freshwater fish, for residues of electron-capturing industrial chemicals that are recovered in the 6% ethyl ether–petroleum ether eluate of the Florisil cleanup procedure (sec. **29.015** (1)), but are too volatile for electron capture gas chromatography (EC/GC) analysis at the operating conditions recommended in sec. **29.018** (1). EC/GC of these volatile compounds, called "early eluting industrial chemicals" because they elute from the OV-101 GC column before the residues usually determined by the method, is carried out with the OV-101 column temperature at 130°C instead of the recommended 200°C for pesticides. As part of an ongoing FDA program to identify new or previously unrecognized industrial chemical contaminants of foods, our laboratory investigates food samples that give unidentified analytical responses when monitored for early eluting industrial chemicals at FDA field laboratories.

In one of these investigations, monochloro- and dichloronitrobenzene residues were identified in a sample of Mississippi River buffalofish caught about 60 miles south of St. Louis, MO. The sample was first noted to yield an unidentified EC/GC response in an analysis for early eluting residues at the FDA Minneapolis District laboratory. When the analytical characteristics of the unknown compound were found to differ from those of the compounds listed in an FDA compilation of GC characteristics and AOAC method behavior data for volatile industrial chemicals, the sample was sent to this laboratory for further study. After the residues were tentatively identified as monochloro- and dichloronitrobenzene congeners by GC/mass spectrometry (MS), retention times and recoveries through the nonfatty food extraction and cleanup procedures of the AOAC method (1) were determined for 15 monochloro- through pentachloronitrobenzene congeners. Follow-up analyses of 12 additional fish samples from the Mississippi River and 6 fish samples from the last 300 miles of the Missouri River were performed. Chloronitrobenzenes were found at levels up to about 1 ppm in 7 samples caught in the Mississippi River near or below St. Louis. Residues found included 2-, 3-, and 4-chloronitrobenzene and 2,3- and 3,4-dichloronitrobenzene; their identities were confirmed by GC/MS comparisons with reference standards of the congeners.

Monochloronitrobenzenes have been reported as contaminants of river and drinking waters (3), but neither these compounds nor dichloronitrobenzenes have previously been reported as environmental contaminants of fish or other foods. Annual United States production of

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monochloronitrobenzenes, chiefly the 2-chloro and 4-chloro isomers, is about 150 million lb; these toxic chemicals serve mainly as starting materials for the production of nitrophenols, nitroanilines, chloroanilines, and other intermediates used to manufacture dyes, pigments, pesticides, rubber chemicals, corrosion inhibitors, and pharmaceuticals (4). Pentachloronitrobenzene (quintozene) and 2,3,5,6-tetrachloronitrobenzene (tecnazene) are registered for use as pesticides in the United States; both occur as residues in foods (5).

The method used for determining the chlorinated nitrobenzene residues in fish is based on the AOAC official method for organochlorine pesticide residues in high-moisture nonfatty foods (1). Residues are extracted from the ground sample with acetonitrile, transferred to petroleum ether, cleaned up by Florisil column chromatography, and analyzed by GC. The procedure differs from the AOAC method as follows: The sample size is reduced so that the total weight does not exceed 50 g and the total fat content does not exceed 2 g; interfering residues such as hexachlorobutadiene (HCBD) are removed from the Florisil column by elution with 100 mL petroleum ether before the usual 200 mL 6 and 15% ethyl ether–petroleum ether eluates are collected; and EC/GC is performed at a column temperature of 130°C. Similar modifications have been used to analyze fish for residues of HCBD, chlorinated norbornene derivatives, and chlorinated benzotrifluorides (6–8).

Experimental

Reagents

(a) *General reagents.*—See sec. **29.002** (1). Solvents and reagents were tested for interferences using the GC parameters described below.

(b) *Reference materials.*—2-Chloronitrobenzene (No. 18,576-0), 4-chloronitrobenzene (No. C5,912-2), 2,3-dichloronitrobenzene (No. D6,820-7), 3,4-dichloronitrobenzene (No. D6,880-0), 2,4-dichloronitrobenzene (No. D6,840-1), 2,3,4-trichloronitrobenzene (No. T5,518-8), 2,4,5-trichloronitrobenzene (No. T5,520-4), 2,3,4,5-tetrachloronitrobenzene (No. T770-5), and pentachlorobenzene (QCB) (No. 13,132-6) were purchased from Aldrich Chemical Co., 940 W St Paul Ave, Milwaukee, WI 53233. 3-Chloronitrobenzene (No. P1100) and 2,4,6-trichloronitrobenzene (No. 7594) were purchased from Eastman Kodak Co., 343 State St, Rochester, NY 14650. 3,5-Dichloronitrobenzene (No. D16190) was obtained from Pfaltz & Bauer, Inc., 375 Fairfield Ave, Stamford, CT 06902. 2,3,5,6-Tetrachloronitrobenzene (EPA/FDA No. 144) and pentachloronitrobenzene (EPA/FDA No. 111) were supplied by the Environmental Protection Agency, Health Effects Research Laboratory, Research Triangle Park, NC 27711. 2,5-Dichloronitrobenzene and 2,6-dichloronitrobenzene were obtained from S. W. Page, Division of Chemistry and Physics, FDA, Washington, DC 20204. Standard solutions of the reference materials were prepared in isooctane. QCB was used as the reference compound for GC relative retention measurements.

Apparatus

(a) *General apparatus.*—See sec. **29.005** (1).

(b) *Gas chromatograph with ^{3}H EC detector.*—As described in sec. **29.008** (1), with the following glass columns: (*1*) 1.8 × 4 mm id, packed with 10% OV-101 on 80–100 mesh Chromosorb W (HP); (*2*) 1.8 m × 4 mm id, packed with 10% OV-101 on 80–100 mesh Chromosorb W (HP) and 15% OV-210 on 80–100 mesh Chromosorb W (HP) (1 + 1). Operating conditions: nitrogen carrier gas ca 120 mL/min; temperatures (°C)—column 130, inlet 150, detector 200; recorder span 5 mV; electrometer sensitivity 1×10^{-9} A for full-scale deflection (FSD) of recorder pen. Nitrogen carrier flow was set to elute QCB in 8–10 min from either column; detector voltage was adjusted to produce 1/2 FSD for 1.5 ng QCB.

(c) *Gas chromatograph with ^{63}Ni constant current EC detector.*—Hewlett-Packard 5730A or Varian 3700 with the following columns: (*1*) 1.8 m × 4 mm id glass, packed with 5% OV-101 on 80–100 mesh Chromosorb W (HP); (2) 1.8 × 4 mm id glass, packed with 5% OV-101 on 80–100 mesh Chromosorb W (HP) and 7.5% OV-210 on 80–100 mesh Chromosorb W (HP) (1 + 1); (3) 25 m × 0.2 mm id OV-101 wall-coated open tubular (WCOT) flexible fused silica capillary. Operating conditions: argon–methane (95 + 5) carrier gas 60 mL/min (columns 1 and 2); nitrogen carrier gas 1 mL/min (column 3) with 20 mL/min split flow and 30 mL/min detector make-up; temperatures (°C)—column 130, inlet 250, detector 300; recorder span 1 mV. Detector attenuation was set to give ca 1/2 FSD for 1.5 ng QCB.

(d) *Combined gas chromatograph–mass spectrometer (electron impact (EI))-data system.*—Varian 1700 gas chromatograph/Finnigan 1015 quadrupole mass spectrometer/Finnigan 6000 data system. The gas chromatograph was coupled to the mass spectrometer through a Gohlke all-glass separator and vacuum diverter valve installed in

the GC detector oven between the column and separator (9). Glass GC column: 1.8 m × 2 mm id, packed with 3% OV-101 on 80–100 mesh Chromosorb W (HP). Operating conditions: helium carrier gas 20 mL/min; temperatures (°C)—column 130, inlet 150, separator 260, transfer line 220, ion source 150; mass spectrometer pressure 2×10^{-5} torr; filament emission 500 μA; preamplifier 10^{-7} A/V; 70 eV primary ionizing voltage in EI mode; scanned mass range m/z 33–350; integration time 6 ms/atomic mass unit; data acquisition under computer control.

(e) *Combined gas chromatograph-mass spectrometer (chemical ionization (CI))-data system.*—Finnigan 9600 gas chromatograph/Finnigan 4023T quadrupole mass spectrometer equipped with pulsed positive ion negative ion (NI) CI option/INCOS 2300 data system. The gas chromatograph was directly coupled to the mass spectrometer ion source through a 25 m × 0.2 mm id OV-101 WCOT flexible fused silica capillary column. Splitless injections were made at the following operating conditions: temperatures (°C)—injector 160, column held at 90° for 1 min after injection, then programmed at 15°/min to 150° and held at 150° for 10 min, separator region 160°, transfer line region 115, ion source 250; helium carrier gas head pressure 10 psi; septum sweep (0.8 min after injection) 40 mL/min; methane reagent gas used to increase source pressure to 0.3 torr; electron energy 70 eV; filament emission 0.25 mA. Samples and reference materials were compared in the NI CI mode using multiple ion detection for ions of m/z 35, 37, 127, 129, 157, 159, 161, 163, 191, 193, and 195 with repetitive 1.2 s scans.

Preparation, Extraction, and Cleanup of Fish

Fish were prepared for analysis in accordance with the edible portion guide of the FDA *Pesticide Analytical Manual* (PAM I) (sec. 141.12c (10)). Before extraction, the edible portion of each sample was thoroughly mixed and ground in a meat grinder as described in *PAM I* (sec. 142.4(5) (10)). Ground fillets of ocean perch were used as the sample substrate in recovery studies. For several fish samples, including ocean perch, the approximate fat content of the edible tissue was determined as in the official fatty food extraction procedure for fish (sec. **29.012(e)** (1)).

Residues were extracted from the homogenized fish samples with acetonitrile by using an adaptation of the official extraction procedure for high-moisture nonfatty foods containing <5% sugar (sec. **29.011(a)**(*1*) (1)). This procedure, which is normally applied to extract 100 g samples of fruits or vegetables, was modified for application to fish of known fat content as described in *PAM I* (sec. 211.13(f)(2) (10)), i.e., by reducing the sample weight so that the total amount of fat was ≤2 g (maximum sample size 50 g). For fish of undetermined fat content, the sample weight used in the nonfatty food extraction procedure was limited to 10–15 g, except for one sample, a carp and sucker fish composite, of which two 20–22 g portions were extracted to obtain enough of the residues for GC/MS analysis.

After the residues were extracted from the fish with acetonitrile, they were transferred to petroleum ether (sec. **29.011(e)** (1)), and cleaned up by Florisil column chromatography. The Florisil cleanup procedure (sec. **29.015** (1)) was used without modification for most of the recovery studies with fortified samples of ocean perch; for other analyses of fish, the procedure was modified to elute potential interfering residues from the Florisil column with 100 mL petroleum ether before the usual 6, 15, and 50% ethyl ether–petroleum ether eluates were collected. Each of the eluates was evaporated to ca 5 mL in a Kuderna-Danish concentrator equipped with a Snyder distilling column. For EC/GC analysis, the volume of each concentrated Florisil eluate was adjusted with petroleum ether so that a 3–8 μL aliquot was equivalent to 20 mg sample. When further concentration of the eluate was required, as for GC/MS analysis, the solvent was evaporated to a suitable volume in a Kuderna-Danish receiver tube equipped with a micro-Snyder column. (Because of the volatile nature of the residues of interest, solvent evaporation under jets of air or nitrogen was avoided.) The 15% ethyl ether–petroleum ether eluates were stored in the dark unless their EC/GC analyses were completed on the same day as the Florisil column cleanup. When these eluates or their concentrates were allowed to stand in normal laboratory light, the "solvent" peaks in their EC chromatograms increased in size as a function of time and became large enough in ca 1 week to obscure the responses for monochloronitrobenzenes.

Gas Chromatography

A 10 μL syringe was used to inject 3–8 μL aliquots of the concentrated sample eluates and reference standard solutions for analysis by EC/GC. Retention times of peaks for residues and standards were measured from the solvent peak front and converted to retention times relative to QCB. Peak height was used as the mea-

sure of EC response. Amounts of residues in the injected aliquots of samples were determined by comparing residue peaks with standard peaks of similar heights within the linear response range of the EC detector. An OV-101 WCOT capillary column was used to determine the level of 3-chloronitrobenzene in a composite of Mississippi River carp and sucker fish. All other residues were quantitated with mixed OV-101 + OV-210 columns. (The OV-101 packed columns were used only for screening and/or confirmatory purposes.)

Results and Discussion

In the analysis of the buffalofish sample at the FDA Minneapolis District laboratory, the AOAC multiresidue method (1) was modified to elute polychlorinated biphenyls from the Florisil column with petroleum ether before the usual 6% ethyl ether–petroleum ether eluate was collected. EC/GC of the petroleum ether eluate on OV-101 and OV-101 + OV-210 columns at 130°C gave chromatograms with a major peak at the retention time of HCBD on each column. Examination of the 6% mixed ether eluate at the EC/GC conditions revealed an unknown compound that eluted from the OV-101 column at about the retention time of HCBD, but that eluted from the OV-101 + OV-210 column at more than twice the retention time of HCBD. In accordance with FDA monitoring program instructions for findings unidentified early eluting residues in foods, a portion of the homogenized buffalofish sample and related analytical information were sent to our laboratory for further study.

Our analysis of the sample of buffalofish was performed as described under *Experimental.* The EC chromatograms of the petroleum ether and 6% ethyl ether–petroleum ether eluates were very similar to those obtained at the Minneapolis laboratory. GC/EI-MS was used to examine both eluates; the MS data confirmed the identity of the HCBD in the petroleum ether eluate and suggested that the residue in the 6% mixed ether eluate was a monochloronitrobenzene. GC retention times were determined for the 3 monochloronitrobenzene isomers; only the 2-chloro isomer eluted from the OV-101 and mixed OV-101 + OV-210 columns at the same retention times as the residue in the 6% mixed ether eluate. GC/EI-MS comparison with the reference standard verified the identification of the residue as 2-chloronitrobenzene.

After the residue was identified, samples of ocean perch were fortified with 2-chloronitrobenzene and analyzed using the same analytical

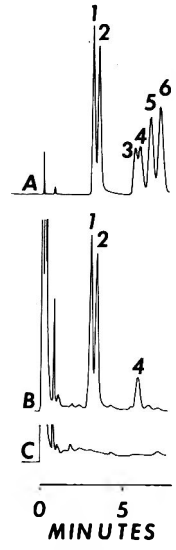

Figure 1. EC (^{63}Ni) gas chromatograms of A, 15% ethyl ether–petroleum ether Florisil eluate of Mississippi River buffalofish (23 mg sample equivalent injected); B, mixture of reference compounds: (1) 1.4 ng 4-chloronitrobenzene, (2) 2.3 ng 2-chloronitrobenzene, (3) 1.0 ng 2,4-dichloronitrobenzene, (4) 1.0 ng 3,4-dichloronitrobenzene, (5) 2.5 ng 2,3-dichloronitrobenzene, and (6) 0.44 ng 2,4,6-trichloronitrobenzene; and C, 15% ethyl ether–petroleum ether eluate of ocean perch (15 mg sample equivalent injected). Mixed OV-101 + OV-210 column with GC conditions in *Apparatus* (c).

procedure as was used for the buffalofish sample. The results of this recovery study, in which petroleum ether was used as the first eluting solvent in the Florisil column cleanup, showed that the compound eluted in both the 6 and 15% mixed ether eluates, with the bulk of it in the latter.

Examination of the 15% mixed ether eluate derived from the buffalofish revealed additional 2-chloronitrobenzene and 2 other residues, subsequently identified as 4-chloronitrobenzene and 3,4-dichloronitrobenzene. Although the 15 and 50% mixed ether Florisil eluates of the AOAC method are often examined for early eluting compounds as part of this laboratory's research on volatile contaminants in foods, these Florisil eluates are not examined for early eluting industrial chemicals under present FDA food surveillance programs. Consequently, the 4-chloronitrobenzene and 3,4-dichloronitrobenzene residues in the buffalofish were not detected in the original analysis of the sample.

Figure 1 shows EC/GC curves obtained by using a mixed OV-101 + OV-210 column for analysis of the 15% mixed ether eluate of the

Table 1. Relative retention times [a] of chlorinated nitrobenzenes on packed GC columns [b]

Chlorinated nitrobenzene	OV-101	OV-101 + OV-210
3-Chloro	0.23	0.41
4-Chloro	0.25	0.43
2-Chloro	0.25	0.48
3,5-Dichloro	0.37	0.56
2,6-Dichloro	0.40	0.71
2,5-Dichloro	0.42	0.70
2,4-Dichloro	0.45	0.79
3,4-Dichloro	0.50	0.83
2,3-Dichloro	0.51	0.94
2,4,6-Trichloro	0.64	1.00
2,4,5-Trichloro	0.85	1.33
2,3,4-Trichloro	1.03	1.70
2,3,5,6-Tetrachloro	1.37	1.92
2,3,4,5-Tetrachloro	1.80	2.57
Pentachloro	2.97	3.83

[a] Relative to QCB.
[b] Columns: 5% OV-101; 5% OV-101 + 7.5% OV-210 (1 + 1). GC parameters given in *Apparatus* (**c**).

buffalofish, a mixture of chlorinated nitrobenzene standards, and the 15% mixed ether eluate of a control sample of ocean perch. The residues indicated by peaks 1 and 2 in the chromatogram of the buffalofish (Figure 1A) eluted from the mixed OV-101 + OV-210 column at the same retention times as 4-chloronitrobenzene and 2-chloronitrobenzene, respectively. When these residues and the corresponding standards (peaks 1 and 2, Figure 1B) were chromatographed on OV-101 columns at 130°C, all eluted at the same retention time. GC/EI-MS comparison of the co-eluting residues with the individual monochloronitrobenzene standards, in combination with the EC/GC retention data, demonstrated that the 15% mixed ether eluate of the buffalofish contained both the 2- and 4-chloronitrobenzene isomers.

The EI mass spectrum of the residue that produced peak 4 in Figure 1A was tentatively identified as that of one or more dichloronitrobenzene isomers. To determine the identity of the specific dichloro isomer(s) and to determine whether other chlorinated nitrobenzenes were present in the sample, the GC characteristics of 15 chloronitrobenzene congeners and the behavior of these compounds in the AOAC nonfatty food extraction and cleanup procedures were investigated.

Table 1 lists the congeners studied and their GC retention times relative to QCB on OV-101 and mixed OV-101 + OV-210 columns at 130°C. (The reported relative retention data are for the columns described in *Apparatus* (c); virtually the same values were obtained with the more heavily loaded columns described in *Apparatus* (**b**).) The EC responses (peak heights) for the chloronitrobenzenes approximated that of an equal amount of QCB; the ^{63}Ni detector provided about 2–3 times greater response for the chlorinated nitrobenzenes relative to QCB than the ^{3}H detector. The GC retention data for 2 columns indicated that the dichloronitrobenzene isomer in the buffalofish (peak 4, Figure 1A) was 3,4-dichloronitrobenzene. GC/EI-MS comparison of the residue with the standard confirmed this finding.

Table 2. Recovery of chlorinated nitrobenzenes from ocean perch by AOAC pesticide multiresidue extraction/cleanup procedures for nonfatty foods [a]

Chlorinated nitrobenzene	Added, ppm	Rec., %	Eluate [b]
2-Chloro	0.50	69, 71	6, 15
	0.050	68, 74	
3-Chloro	0.42	71	15
	0.042	82	
4-Chloro	0.80	80, 80	15
	0.080	79, 83	
2,3-Dichloro	0.25	83, 95	6, 15
	0.025	87, 92	
2,4-Dichloro	0.20	89, 95	6, 15
	0.020	90, 97	
2,5-Dichloro	0.16	104, 116	6
2,6-Dichloro	0.11	88, 101	6
3,4-Dichloro	0.20	86, 90	15
	0.020	87, 92	
3,5-Dichloro	0.11	88, 107	6, 15
2,3,4-Trichloro	0.24	97, 103	6, 15
	0.024	93, 95	
2,4,5-Trichloro	0.45	95, 102	6, 15
	0.045	100, 102	
2,4,6-Trichloro	0.48	101, 102	6
	0.048	103, 103	
2,3,4,5-Tetrachloro	0.36	99, 104	6, 15
	0.036	95, 99	
2,3,5,6-Tetrachloro	0.37	86, 102	6
	0.037	101, 104	
Pentachloro	1.00	84, 102	6
	0.100	100, 103	

[a] Nonfatty food extraction cleanup (secs. **29.011**(**a**)(*1*) and (**e**) and **29.015** (1). Extraction modified for fish as in *PAM I* (sec. 211.13(f)(2) (10)).
[b] Florisil eluate containing compound: 6 = ethyl ether–petroleum ether (6 + 94); 15 = ethyl ether–petroleum ether (15 + 85).

In studies on the behavior of the chloronitrobenzenes in the AOAC nonfatty food method, the Florisil cleanup procedure (sec. **29.015** (1)) was used without the modification made for the analysis of the Mississippi River buffalofish, i.e., the usual 6, 15, and 50% mixed ether eluates were *not* preceded by a 100 mL petroleum ether eluate. (The elution with petroleum ether was omitted so that the recovery data would show the eluates in which the compounds would normally be recovered.) Table 2 lists the recoveries of 15

chloronitrobenzenes from fortified samples of ocean perch and the Florisil column eluate(s) in which each compound was recovered. The recovery values for compounds that split between the 6 and 15% mixed ether eluates were calculated by adding the amounts found in both eluates. No chloronitrobenzenes were found in the 50% mixed ether eluates of the spiked samples. The data in Table 2 indicate that the analytical procedure recovers a smaller portion of the monochloronitrobenzenes (68–83%) than of the dichloro through pentachloro congeners (83–116%); this may reflect losses of the relatively more volatile monochloro compounds in the solvent evaporation steps of the procedure. Based on EC/GC analyses of Florisil eluate aliquots equivalent to 20 mg sample, the method provides chloronitrobenzene residue quantitation limits of about 0.005 and 0.025 ppm with ^{63}Ni and ^{3}H EC detectors, respectively.

Further work with the modified Florisil cleanup procedure used in the analysis of the buffalofish sample has shown that elution of the Florisil column with 100 mL petroleum ether does not recover any of the chloronitrobenzenes listed in Table 2, but may affect the relative amounts of the compounds recovered in the succeeding 6 and 15% mixed ether eluates. The particular lot of Florisil and the total amount of fat applied to the Florisil column also may affect the degree to which these compounds split between the 6 and 15% mixed ether eluates; thus the Florisil elution behavior of the chloronitrobenzenes may vary from that presented as a general guide in Table 2.

To determine whether chloronitrobenzenes as well as the 3 previously identified congeners were present at ≥0.005 ppm in the Mississippi River buffalofish, the EC chromatograms of the 4 Florisil eluates of the sample were examined for responses at the retention times listed in Table 1. The chromatograms of the 15% mixed ether eluate suggested the presence of a residue at the retention time of 3-chloronitrobenzene, as indicated by a slender shoulder at the front of peak 1 (4-chloronitrobenzene) in the OV-101 + OV-210 chromatogram (Figure 1A) and by a similar shoulder at the front of the peak for the co-eluting 2- and 4-chloronitrobenzenes in the OV-101 column chromatogram. For comparison with the sample residues, standard solutions containing 1 part of 3-chloronitrobenzene and 1, 5, or 10 parts of 4-chloronitrobenzene were chromatographed on the OV-101 + OV-210 column (*Apparatus* (c)). The responses for 3-chloronitrobenzene in the resulting chromatograms appeared as the first of 2 overlapping peaks (separated by a valley) for the 1 + 1 mixture, as a discrete shoulder at the front of the major peak for the 1 + 5 mixture, and as a slender shoulder at the front of the major peak for the 1 + 10 mixture. Although the latter shoulder was quite narrow, it was more distinct than the shoulder at the retention time of 3-chloronitrobenzene in the chromatogram of the 15% mixed ether eluate of the buffalofish (Figure 1A). Thus, if 3-chloronitrobenzene was present as a residue in the buffalofish, its indicated concentration was less than 10% of that of the 4-chloronitrobenzene residue. No additional EC/GC responses attributable to chlorinated nitrobenzenes other than the monochloro and 3,4-dichloro congeners were detected in the buffalofish chromatograms.

Upon completion of the buffalofish sample analysis, 18 additional samples of fish from the Mississippi and Missouri Rivers were examined for chloronitrobenzene residues (Table 3). All of the residues were found in fish caught in the Mississippi River near St. Louis or in a 150 mile section of the river south of that city. No chloronitrobenzene residues (<0.005 ppm) were detected in 2 samples of Mississippi River fish collected 100 miles north of St. Louis, 3 samples collected 260–400 miles south of St. Louis, or 6 samples of Missouri River fish. The highest chloronitrobenzene residue levels were found in a composite of carp and sucker fish caught near a chemical waste disposal site at Sauget, IL, a city just across the Mississippi River from St. Louis. EC/GC with both packed columns (*Apparatus* (c)) indicated that the chloronitrobenzene residues in this sample could include the 3-chloro and 2,3-dichloro congeners in addition to the 3 compounds previously confirmed as residues in the buffalofish. Because the packed GC columns did not provide adequate resolution for quantitation of 3-chloronitrobenzene in the presence of larger amounts of 4-chloronitrobenzene and because only one of the columns (OV-101 + OV-210) resolved the 2- and 4-chloro isomers or the 2,3- and 3,4-dichloro isomers, capillary column EC/GC was investigated for analysis of the residues.

A 25 m OV-101 WCOT fused silica capillary column provided adequate resolution of all the monochloro- and dichloronitrobenzenes. Figure 2 shows the chromatograms obtained for injections of the concentrated 6% ether eluate of the Sauget fish composite and a mixture of standard solutions containing the 3 monochloro- and 6 dichloronitrobenzenes. The residues were

Table 3. Chlorinated nitrobenzenes (ppm) found in edible portion of fish from Mississippi River

Species	Origin[a]	2-Chloro	3-Chloro	4-Chloro	2,3-Dichloro	3,4-Dichloro
Catfish	0	[b]	[b]	0.008	[b]	[b]
Catfish	0	0.064	[c]	0.19	[b]	[b]
Catfish	0	0.053	[c]	0.088	[b]	[b]
Catfish	0	0.026	[c]	0.20	[b]	[b]
Carp/sucker[d]	0	0.24	0.057[e]	0.63	0.024	0.085
Buffalofish	60[f]	0.12	[c]	0.20	[b]	0.03
Catfish	150[g]	0.006	[b]	0.019	[b]	[b]
Catfish	150[g]	0.027	[b]	0.025	[b]	[b]

[a] Fish collection site, in miles south of St. Louis.
[b] Not detected, limit of quantitation <0.005 ppm.
[c] GC resolution for analysis was inadequate to detect or determine 3-chloronitrobenzene in presence of 10-fold larger amounts of 4-chloronitrobenzene. If 3-chloro isomer is present in sample, its concentration is estimated as <10% of that of 4-chloro isomer.
[d] Composite of 2 species.
[e] Residue value determined using WCOT capillary GLC column.
[f] Near Kimswick, MO.
[g] Near Cape Girardeau, MO.

found at the retention times of the 2-chloro-, 3-chloro, 4-chloro-, 2,3-dichloro-, and 3,4-dichloronitrobenzenes. The presence of these 5 congeners in the 6% mixed ether eluate of the Sauget fish sample was confirmed by comparing the ion current chromatograms obtained for the residues

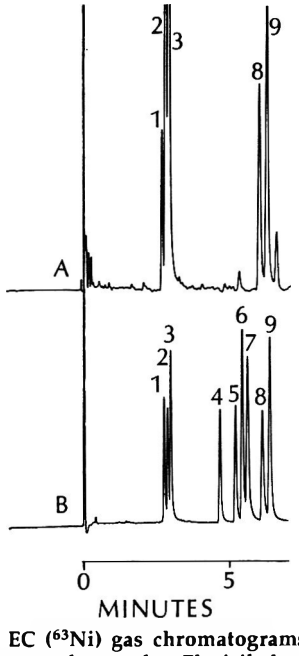

Figure 2. EC (^{63}Ni) gas chromatograms of A, 6% ethyl ether-petroleum ether Florisil eluate of carp/sucker composite (10.5 mg sample equivalent injected); B, mixture of chlorinated nitrobenzene congeners: (1) 3-chloro, (2) 4-chloro, (3) 2-chloro, (4) 3,5-dichloro, (5) 2,6-dichloro, (6) 2,5-dichloro, (7) 2,4-dichloro, (8) 3,4-dichloro, and (9) 2,3-dichloro. OV-101 WCOT capillary column with GC conditions in *Apparatus* (c).

and reference standards by using capillary GC/MS in the NI CI mode with multiple ion detection of ions characteristic of monochloro- and/or dichloronitrobenzenes (*Apparatus* (e)).

EC/GC with the ^{63}Ni detector and the mixed OV-101 + OV-210 column described in *Apparatus* (c) was used to determine the residue levels reported in Table 3, except for 3-chloronitrobenzene, which was determined by using the OV-101 WCOT capillary column. Quantities of compounds found in both the 6 and 15% mixed ether eluates were combined for reporting in Table 3. As shown by Figure 2, the 6% mixed ether eluate of the Sauget fish composite contained 3 compounds which would normally be recovered in the 15% mixed ether eluate, viz., 3-chloro-, 4-chloro-, and 3,4-dichloronitrobenzene. Additional amounts of these compounds (and 2-chloronitrobenzene) were found in the 15% mixed ether eluate; however, this eluate did not contain 2,3-dichloronitrobenzene, a compound which split between the 6 and 15% mixed ether eluates in recovery studies with fortified ocean perch. Although the same lot of Florisil was used as in the recovery studies, the aberrant residue elution pattern was observed for both of the 20–22 g portions of the Sauget fish composite that were processed to obtain enough of the residues for GC/MS analysis. Since the fat content of the composite was not determined, the portions taken for analysis may have contained more than 2 g fat and this, either by itself or in combination with the modification of the Florisil cleanup procedure to use the 100 mL petroleum ether eluate, may have affected the Florisil elution behavior of the residues.

The residue findings reported here indicate

that lower chlorinated nitrobenzenes are contaminants of the 150 mile section of the Mississippi River extending from St. Louis to Cape Girardeau, MO. The examination of fish for these and other of the more volatile chemical contaminants by this laboratory is continuing.

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Determination of Coumaphos and Its Oxygen Analog in Eggs and Milk by Using a Multiresidue Method with Liquid Chromatographic Quantitation and Capillary Gas Chromatographic/Mass Spectrometric Confirmation

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A multiresidue method for carbamate insecticides was adapted for the determination of coumaphos and its oxygen analog in eggs and milk. Eggs were extracted with acetonitrile and milk was extracted with acetone. Co-extractives were removed using liquid partitioning and charcoal column procedures described in the carbamate method. Coumaphos and its oxygen analog were determined by using a high performance liquid chromatograph equipped with a fluorescence detector. Recovery studies were performed for the 2 compounds at levels of 0.01 and 0.10 ppm in eggs and 0.01 and 0.02 ppm in milk. Overall average recovery was 100% (range 95-109%). In a trial of the method by another laboratory, the recovery of coumaphos and its oxygen analog from milk averaged 87 and 96%, respectively. Data are presented on the capillary gas chromatographic/mass spectrometric confirmation of coumaphos residues.

Coumaphos [*O,O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-(2*H*)-1-benzopyran-7-yl) phosphorothioate] is registered for use as a topical insecticide to control flies, fleas, and lice on livestock and poultry (1, 2). Coumaphos can also be added to feed to control roundworms in cattle and poultry (3). The tolerance in the United States for residues of coumaphos and its oxygen analog metabolite is 0.5 ppm in milk fat and 0.1 ppm in eggs (2).

The determination of coumaphos residues has been reported using fluorometric or gas chromatographic (GC) techniques. Thornton and Anderson (4) determined coumaphos and coumaphos oxygen analog in eggs by using a gas chromatograph with a packed column of Gas-Chrom Q coated with DC-200, and a thermionic detector. Bowman et al. (5) used a gas chromatograph with a flame photometric detector to determine coumaphos and its oxygen analog in milk. The 2 compounds were separated by Bowman et al. before GC because they were not adequately separated on the GC column containing the DC-200 liquid phase. This laboratory has also observed inadequate separation of the 2 compounds with the DC-200 column. Also, the appreciably lower response of the detector to the oxygen analog vs the response for the parent was mutually observed. Other GC liquid phases (DEGS, OV-17, and SP-1240DA) examined in this laboratory were inadequate for separating coumaphos and its oxygen analog.

Adams and Anderson (6) determined coumaphos residues in milk by measuring the fluorescence of chlorferron, a hydrolysis product of coumaphos and its oxygen analog. Zakrevsky and Mallet (7) reported the individual determination of coumaphos and its oxygen analog in eggs by measuring their natural fluorescence directly on a thin layer plate.

Liquid chromatography (LC) has been used to separate a number of naturally fluorescent pesticides (8) and appeared promising as a determinative technique for coumaphos and coumaphos oxygen analog. This technique was used in a multiresidue method for *N*-methylcarbamate insecticides and several naturally fluorescent pesticides (9, 10). Methanol was used to extract the residues and liquid-liquid partitioning and charcoal adsorbent removed the crop co-extractives before LC determination.

The purpose of the work reported here was to examine the applicability of reverse phase LC for the separation and fluorometric detection of coumaphos and its oxygen analog and to ascertain the applicability of the multiresidue method for *N*-methylcarbamate insecticides and other pesticides (9, 10) to the determination of both compounds. In addition, confirmation of the presence of coumaphos residues in eggs and milk was investigated using a capillary gas chromatograph coupled to a mass spectrometer.

METHOD

Coumaphos and coumaphos oxygen analog are extracted from eggs using acetonitrile and from milk using acetone. Co-extractives are removed using liquid-liquid partitioning and a charcoal-silanized Celite adsorbent mixture. Coumaphos and its oxygen analog are determined

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using a liquid chromatograph equipped with a C-8 column and a fluorescence detector. This LC method is an adaptation of the multiresidue method for carbamate insecticides (9). The compound identity is confirmed using a capillary gas chromatograph coupled to a mass spectrometer.

The carbamate method (9) was used in the study with the following additions and changes.

Reagents

Acetone.—Distilled-in-glass grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442).

Reagents (d), (f), and (g) (9) are not needed.

LC Operating Parameters

Adjust mobile phase flow rate to 1.50 ± 0.02 mL/min at 50% acetonitrile in water. Equilibrate system at 12% acetonitrile in water for 10 min, inject sample, and begin 30 min linear gradient to 70% acetonitrile in water, and then 100% acetonitrile for additional 5 min. Operate column oven at 35°C. Maintain hydrolysis chamber at ambient temperature. Set fluorescence detector excitation and emission wavelengths at 278 and 306 nm, respectively. Set detector photomultiplier gain at low and time constant at 1 s. Adjust sensitivity so that 50 ng carbofuran produces 50% full scale response on printer plotter (recorder). For determination of coumaphos and coumaphos oxygen analog, use excitation and emission wavelengths of 320 and 385 nm, respectively.

GC/Mass Spectrometric (MS) Apparatus

(a) *Gas chromatograph.*—Finnigan Model 9610, interfaced to Finnigan 4023T mass spectrometer. A 50 m × 0.20 mm id open tubular fused silica capillary column, wall-coated with SE-30 and connected directly to the ion source, was used. GC oven temperature was programmed: 60°C for 1 min and increased to 270°C at 20°/min. Injector temperature was maintained at 200°C. Carrier gas was helium; flow rate was ca 25 cm/s.

(b) *Mass spectrometer.*—Finnigan Model 4023T, and Finnigan 2300 data system with revision 3.98 software. Mass spectrometer was operated in multiple ion detection mode using electron ionization (EI). Major ions monitored were m/z 109, 125, 210, 226, and 362 of coumaphos (MW = 362) and 109, 182, 210, 220, 290, 318, and 346 of oxygen analog (MW = 346). Emission current was 0.25 mA and electron energy 70 eV from heated rhenium filament. Ion source was maintained at 250°C.

Extraction

Eggs.—Same as for high moisture products, except extract eggs with acetonitrile. Also, add 0.5 mL hexadecane to round-bottom (r-b) flask before concentrating extract to reduce foaming. Continue with carbamate method (9) beginning, "Place 250 mL ⅋ 24/40 trap on 2 L r-b flask . . ."

Milk.—Add 150 g milk and 300 mL acetone to 500 mL centrifuge bottle. Using rubber stopper wrapped in acetone-washed aluminum foil, stopper centrifuge bottle and vigorously shake 1 min. Centrifuge 5 min at 1200 rpm. Decant 277 mL supernate (equivalent to 100 g milk) into 500 mL graduated cylinder and transfer to 2 L r-b flask. Add 15 mL distilled or ultrapure water and star magnet to r-b flask. Continue with carbamate method (9) beginning, "Place 250 mL ⅋ 24/40 trap on 2 L r-b flask . . ."

Co-extractive Removal

Follow *Partitioning* and *Chromatographic* steps as given in carbamate method (9).

Determination

Inject 10 μL methanol sample solution onto LC column, using chromatographic apparatus and parameters as described. Tentatively identify residue peaks on basis of retention times. Measure peak area or peak height and determine amount of residue by comparison to peak area or peak height obtained from known amount of appropriate reference materials(s). To ensure valid quantitation of residue, size of peaks from sample residue and reference standard should match within ± 25%. Chromatograph reference materials(s) immediately after sample.

Confirmation

A variety of techniques may be useful for confirmation of residue identity, as considered necessary. In this work, confirmation was obtained by GC/MS according to the following procedure:

Concentrate portion of methanol sample solution so that 1 μL contains 2 ng coumaphos or 20 ng coumaphos oxygen analog. Inject 1 μL concentrated sample solution onto GC capillary column. Vent solvent 1 min. Acquire spectra between 20 and 25 min. Compare sample spectra to those of coumaphos and coumaphos oxygen analog obtained in same manner and at same retention.

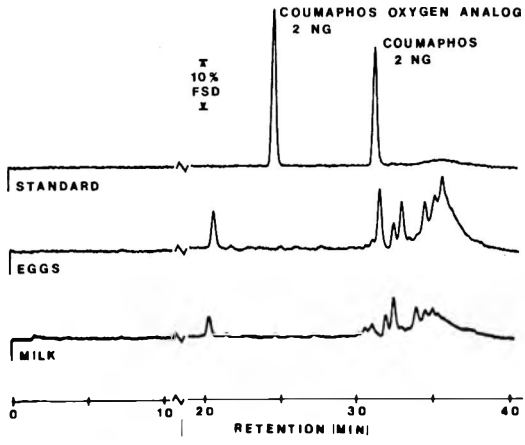

Figure 1. Chromatograms of coumaphos and oxygen analog standards, egg control, and milk control.

Results and Discussion

LC-Fluorescence Studies

The fluorescent characteristics of coumaphos and coumaphos oxygen analog were studied in acetonitrile–water (1 + 1) and acetonitrile–aqueous 0.04N NaOH (1 + 1), using the LC fluorescence detector at static conditions. Both compounds fluoresced in the neutral solution but not in the basic solution. The maximum excitation and emission wavelengths were 320 and 380 nm, respectively, for coumaphos and 320 and 388 nm, respectively, for coumaphos oxygen analog. Therefore, an excitation wavelength of 320 nm and an emission wavelength of 385 nm were selected for the determination of both compounds.

Utilizing the LC parameters under *Method*, which were previously used for other fluorescent pesticides (10), retention and response data for coumaphos and coumaphos oxygen analog were obtained. Figure 1 shows an LC chromatogram of coumaphos and its oxygen analog, which elute in 31 and 24 min, respectively. Five ng coumaphos produces a peak of approximately 50% full scale deflection (FSD), and the linear response range is from 0.5 to 14 ng. Four ng coumaphos oxygen analog produces a peak of approximately 50% FSD, and the linear response range is from 0.5 to 12 ng. Of 38 other pesticides/metabolites that fluoresce naturally in neutral solution (8), none have the same fluorescence or LC retention properties as the 2 coumaphos compounds. Thus LC, in conjunction with the fluorescence detector, provides the sensitivity and selectivity desired in a pesticide residue method.

Multiresidue Method Studies

The multiresidue method for determining *N*-methylcarbamate insecticides (9) uses methanol and a Polytron homogenizer to extract the pesticides from crops. Methanol does not adequately precipitate egg or milk solids, which results in the formation of severe emulsions during liquid–liquid partitioning. Two other solvents, acetone and acetonitrile, were tested for extraction of coumaphos residues from eggs and milk, and both solvents appeared to precipitate egg and milk solids well. For extracting eggs in a Polytron homogenizer, acetonitrile was the solvent of choice, because only 2% of the liquid was lost by evaporation compared with the 15% volume loss when acetone was used as the extraction solvent. Since the determination is based on an aliquot of the extract, the significant loss of acetone would result in high recoveries of the 2 compounds.

When milk was extracted with acetonitrile, high recoveries were obtained for coumaphos and its oxygen analog. This may have been due in part to the formation of 2 liquid phases which could have disrupted the homogeneity of the extract. With acetone as the extraction solvent, only one liquid phase was observed in the extract. To prevent evaporation of acetone, the milk was extracted with the solvent by shaking the mixture in a stoppered centrifuge bottle. The mixture was centrifuged and a portion of the supernate was decanted into a graduated cylinder. Acetone appeared to be the best solvent as only one extract layer was formed, and no loss of extract occurred.

The egg and milk extracts were concentrated with the rotary evaporator system used for crops (9). The egg extracts required the addition of 0.5 mL hexadecane to minimize frothing during this concentration step. The liquid partitioning and chromatographic co-extractive removal steps of the carbamate method (9) worked well for both egg and milk samples.

Recovery Studies

Studies were conducted to determine the recovery of coumaphos and coumaphos oxygen analog from eggs and milk through the method. Eggs were fortified with both coumaphos and its oxygen analog at the 0.01 and 0.10 ppm levels. Raw milk was fortified with the 2 compounds at the 0.01 and 0.02 ppm levels on a whole product basis. Duplicate recovery determinations were made along with a product control (Table 1). The overall average recovery was 100% with a range from 95 to 109%. The equivalent of 200

Table 1. Recovery (%) of coumaphos and coumaphos oxygen analog from eggs and milk

Added, ppm	Coumaphos	Coumaphos oxygen analog
	Eggs	
0.01	106, 109	97, 99
0.10	102, 102	101, 102
	Milk[a]	
0.01	102, 99	100, 96
0.02	96, 98	95, 98

[a] Whole product basis.

Table 2. Recovery (%) of coumaphos and coumaphos oxygen analog from milk: interlaboratory study

Added, ppm[a]		Coumaphos	Coumaphos oxygen analog
0.012		92	100
0.015		81	93
0.023		87	96
0.031		87	94
	Av.	87	96

[a] Whole product basis.

mg of each product control and product fortified at the 0.01 ppm level was injected onto the LC column. Chromatograms of the product controls are shown in Figure 1. No interfering peaks were observed in eggs or milk near the retention time of coumaphos oxygen analog. However, with the egg control sample a peak of approximately 10% FSD eluted 10 s after coumaphos. The effect of this peak on coumaphos recovery values was minimized by using peak height measurements for quantitation.

In a trial of the method by another laboratory, raw milk samples were fortified with coumaphos and its oxygen analog at from 0.012 to 0.031 ppm on a whole product basis. The individual recovery values are shown in Table 2. The average recoveries of coumaphos and its oxygen analog were 87 and 96%, respectively. The analyst encountered no difficulties. He reported that a 67% acetonitrile in water isocratic mobile phase could be used as an alternative to the gradient system. With the isocratic mobile phase, retention times for coumaphos and its oxygen analog were 8.4 and 3.8 min, respectively; good peak shape was obtained for both compounds. No interfering peaks were observed.

GC/MS Confirmation

Since adequate separation of coumaphos and its oxygen analog is difficult to obtain with packed GC columns, capillary GC was investigated with the GC/MS technique. The fused silica capillary column coated with SE-30 produced retention times of 21 min 52 s and 22 min 45 s for coumaphos oxygen analog and coumaphos, respectively. The column provided adequate separation of the 2 compounds, which had peak widths of 10 s at the baseline.

Eight ions of coumaphos and 12 ions of the oxygen analog were monitored by a quadrupole mass spectrometer operated in the EI mode to provide a high degree of confidence to the proper identity of these compounds. The mass spectrometer could detect 2 ng coumaphos and 20 ng coumaphos oxygen analog. The poorer detectability of the oxygen analog may be due in part to GC on-column degradation. Typical examples of multiple ion EI mass spectra of the 2 compounds obtained from standard solutions and the compounds recovered from fortified eggs are shown in Figures 2 and 3. Similar mass

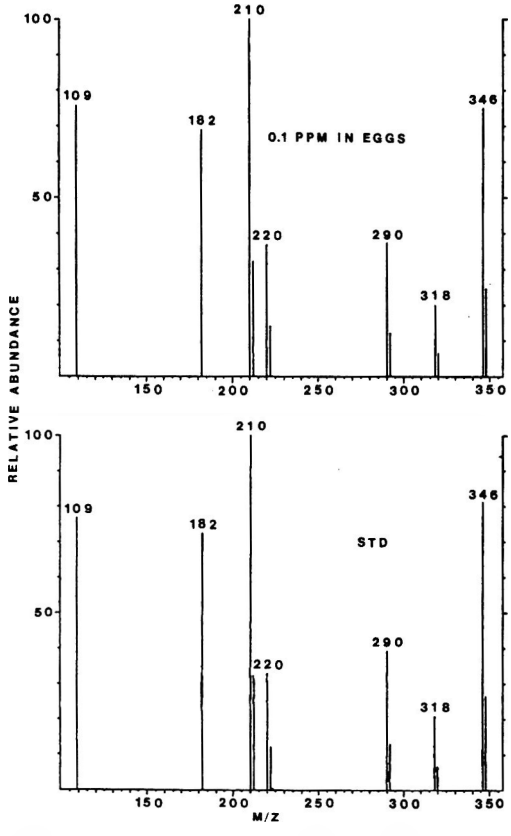

Figure 2. Mass spectra of coumaphos oxygen analog (major ions).

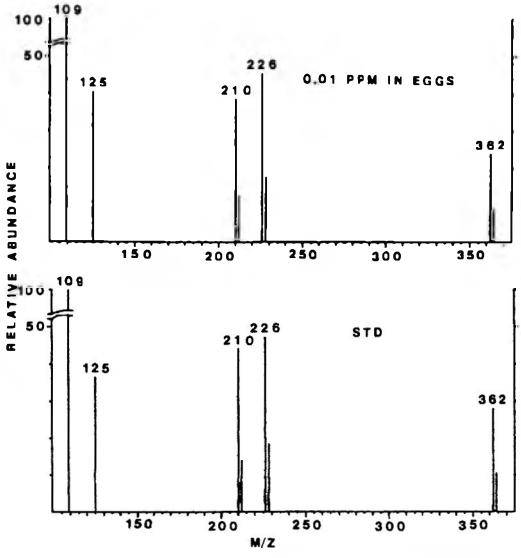

Figure 3. Mass spectra of coumaphos (major ions).

spectra were obtained for coumaphos and its oxygen analog recovered from milk fortified at the 0.02 ppm level. Co-extractives prevented confirming the presence of coumaphos oxygen analog at the 0.01 ppm level in eggs and milk and coumaphos at the 0.01 ppm level in milk. No coumaphos residues were observed in the control samples.

Additional studies have been conducted (11) to enable the confirmation of the 2 compounds at the 0.01 ppm level. In these studies, GC/MS and LC/MS were investigated with the mass spectrometer operated in the chemical ionization mode; both positive and negative ions were monitored.

Conclusions

The multiresidue method for determination of *N*-methylcarbamate insecticides in fruits and vegetables (9) has been successfully adapted for the determination of coumaphos and coumaphos oxygen analog in eggs and milk through modification of the extraction procedure. The presence of both compounds in these products can be confirmed by capillary GC/MS at sub-part-per-million levels.

Acknowledgments

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Comparison of Gas Chromatographic/Mass Spectrometric and Liquid Chromatographic/Mass Spectrometric Methods for Confirmation of Coumaphos and Its Oxygen Analog in Eggs and Milk

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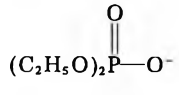
A comparison of a liquid chromatographic (LC)/mass spectrometric (MS) procedure for confirming coumaphos, an organophosphate insecticide, and its oxygen analog in milk and eggs with a capillary gas chromatographic (GC)/MS method is presented. For the confirmation of coumaphos and its oxygen analog, multiple ion detection of both positive and negative ions from the chemical ionization mass spectra was used. Samples of milk and eggs fortified with the 2 compounds at the 0.1, 0.01, and 0.005 ppm levels were analyzed. The major finding is the relatively greater efficiency of the LC/MS interface compared with the GC/MS capillary injector.

Coumaphos, *O,O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-(2*H*)-1-benzopyran-7-yl) phosphorothioate, is a topical insecticide used to control fleas, ticks, and mites on livestock and poultry (1). It is also added to livestock feed as a dewormer. As a result of these applications, investigators have been concerned with coumaphos residues entering the food chain. Both coumaphos and a metabolite (an oxygen analog) have been found in milk (2). During the past



several years considerable effort has been expended on the development and extension of analytical methods for determining organochlorine and organophosphorus pesticides in food. In previous work on food samples, gas chromatography (GC) and GC/mass spectrometry (MS) have been the most frequently used techniques (3, 4). Recently, a liquid chromatographic (LC) procedure has been developed for determining coumaphos and its oxygen analog in milk and eggs with GC/MS as the confirmatory method (5).

A variety of MS techniques have been used to confirm the identities of organophosphorus pesticides. Krause et al. (5) used electron ionization (EI) GC/MS in the confirmatory procedure. The EI behavior of these compounds has been reviewed (6, 7). Parker et al. (8) obtained the negative ion (NI) chemical ionization (CI) mass spectra of 18 organophosphorus pesticides, using a direct liquid insertion probe LC/MS interface. The NI mass spectra obtained in the Parker et al. study were similar to those reported earlier using GC introduction (9). The NI spectra are usually sensitive to the presence of the phosphorus-containing part of the molecule and may also separately indicate the nonphosphorus moiety if it can stabilize a negative charge. In addition, chloride attachment NI mass spectra of organophosphorus pesticides have been reported (10), and the positive ion (PI) CI mass spectra were previously studied (11). The PI CI mass spectra are useful for indicating molecular weight by the relatively abundant $(M + H)^+$ ion, whereas molecular ions ($M^{-\cdot}$) are of low relative abundance in NI CI mass spectra.

In continuing the effort of extending analytical methods, the objective of this study was to develop an LC/MS procedure for confirming coumaphos and its metabolite (oxygen analog) in milk and eggs and to compare the results obtained with those derived from the GC/MS technique. Various approaches to LC/MS are possible. A recent paper (12) reviewed some of the commercially available instrumental literature. The present paper uses PI and NI CI/MS with either the moving belt LC/MS interface or capillary column GC/MS.

Experimental

Sample Preparation

Extracts of milk and eggs were prepared using the basic multiresidue method (13) for *N*-methylcarbamates with the following modifications (5): Milk was extracted with acetone and eggs were extracted with acetonitrile. Three aliquots were taken from each extract. One was spiked with a mixed standard of coumaphos and its metabolite to yield a solution in methanol at the 0.1 ppm level; the other 2 aliquots were first

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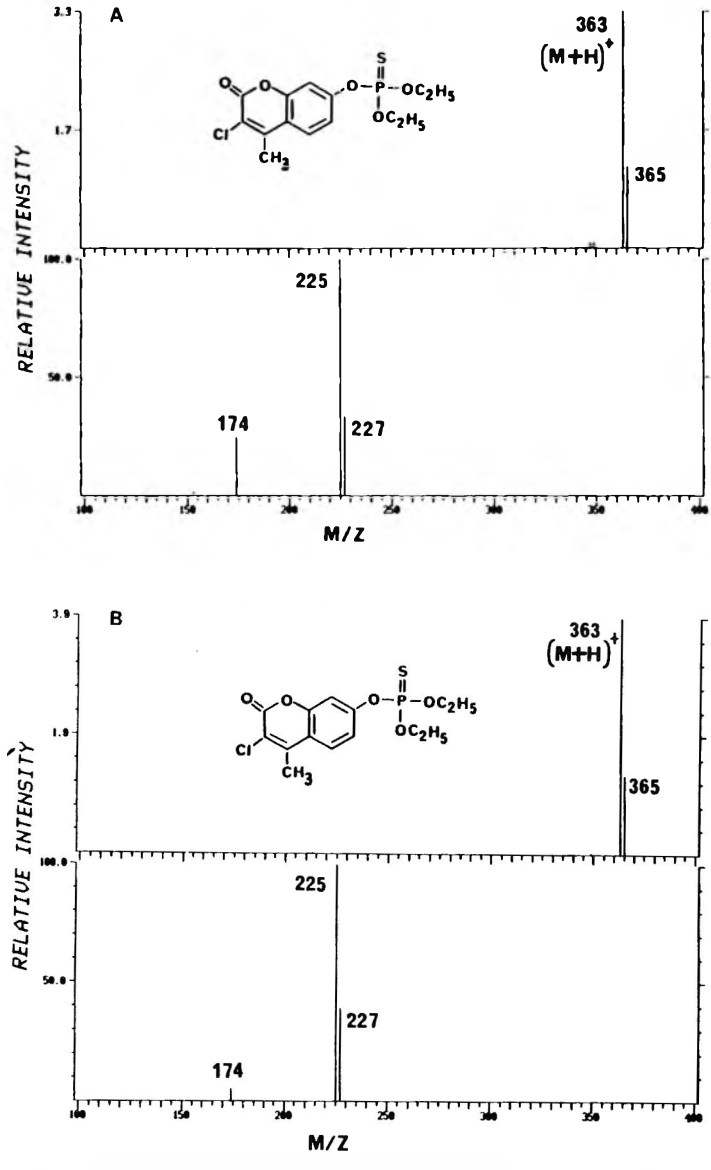

Figure 1. MID CI mass spectra of coumaphos (250°C source, 0.3 torr methane, PI NI). A, GC/MS (4 ng); B, LC/MS (20 ng × 5% split). Upper spectrum, PI; lower spectrum, NI.

concentrated and then spiked to yield solutions at the 0.01 and 0.005 ppm levels, respectively.

Apparatus and Reagents

(a) *Mass spectrometer.*—Finnigan Model 4023T, with Finnigan 2300 data system with revised software. The mass spectrometer was operated in a multiple ion detection (MID) mode using continuous pulse PI NI CI. Positive ions monitored were m/z 363, 365 of coumaphos (MW = 362) and 347, 349 of the oxygen analog (MW = 346). Negative ions monitored were m/z 225, 227, and 174 for coumaphos and m/z 153 and 174 for the oxygen analog. Methane was used as reagent gas at a source pressure of 0.3 torr. Emission current was 0.25 mA and electron energy 70 eV from a heated rhenium filament. Ion source was maintained at 250°C.

(b) *Gas chromatograph.*—Finnigan Model 9610, interfaced to the Finnigan 4023T mass spectrometer. A 25 m × 0.20 mm id open tubular fused silica capillary column, wall-coated with OV-1 and connected directly to the ion source, was used. GC oven temperature was pro-

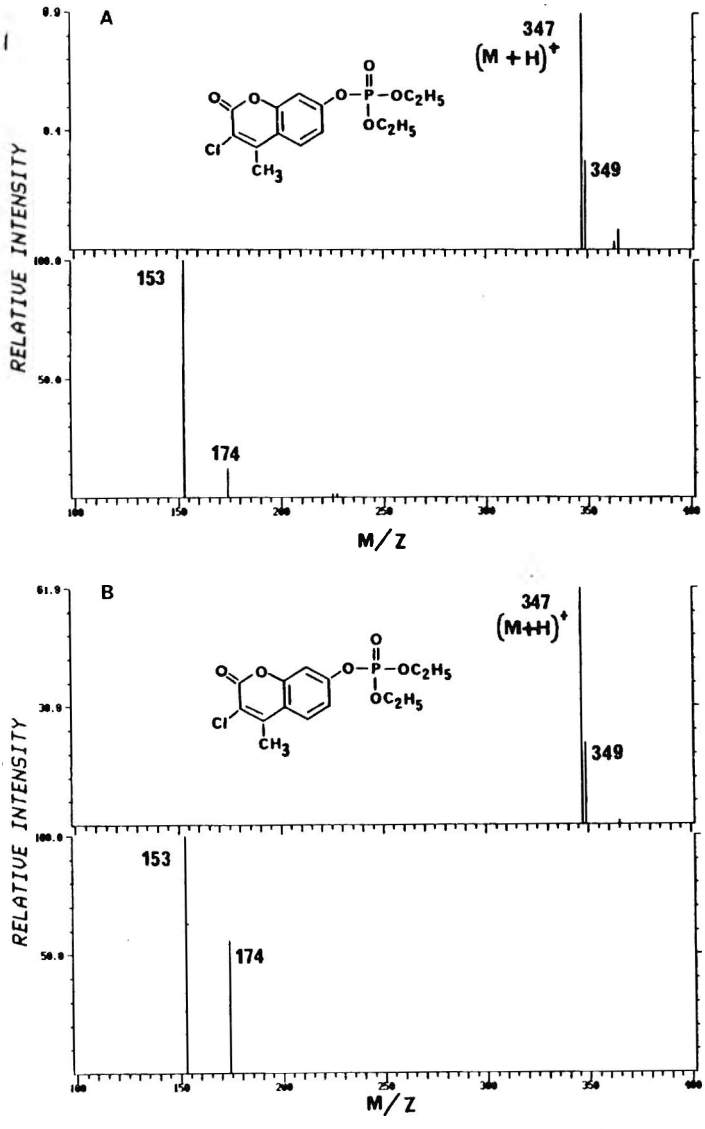

Figure 2. MID CI mass spectra of oxygen analog (250°C source, 0.3 torr methane, PI NI). A, GC/MS; B, LC/MS. Upper spectrum, PI; lower spectrum, NI.

grammed at 60°C for 1 min and increased to 270°C at 20°/min. Injector temperature was maintained at 200°C. Carrier gas was helium; flow rate was ca 25 cm/s.

(c) *Liquid chromatograph.*—DuPont Model 8820 series gradient, with variable wavelength ultraviolet (UV) detector, was interfaced to the Finnigan 4023T mass spectrometer. The interface was a Finnigan moving belt type. A DuPont Zorbax C-8 reverse phase 25 cm × 4.6 mm id column was used. Injections were made with a 10 μL loop. Gradient solvent program: 20% acetonitrile for 2 min, increased to 50% in 10 min, and then increased to 70% in 30 min. Flow rate 1.5 mL/min. Total time 42 min. LC/MS interface: Kapton (polyimide) belt; rotor speed 20 cps; vaporizer 200°C; cleanup heater 200°C; splitter with 5% going to mass spectrometer (20:1 ratio).

(d) *Acetonitrile.*—Distilled-in-glass (Burdick and Jackson).

(e) *Water.*—Distilled, Milli-Q filtered.

Results and Discussion

Although another method for coumaphos confirmation used GC/EI MS (5), the confirma-

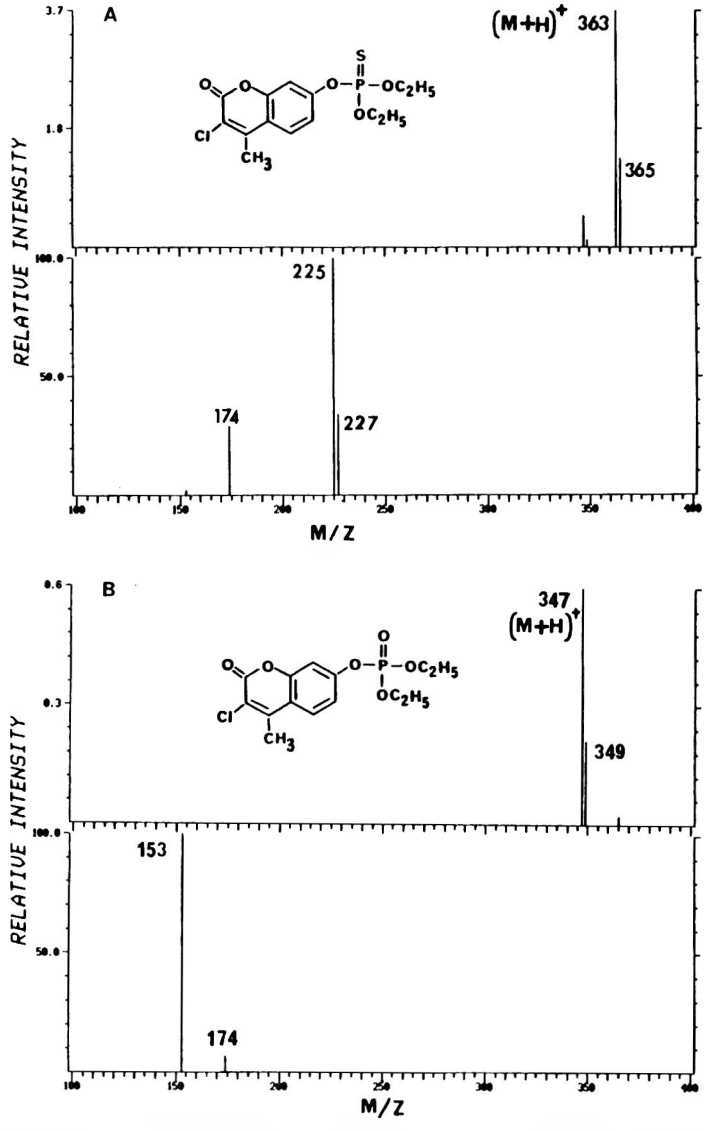

Figure 3. MID (GC/MS) mass spectra at 0.1 ppm level (250°C source, 0.3 torr methane, PI NI). A, coumaphos in eggs; B, oxygen analog in milk. Upper spectrum, PI; lower spectrum, NI.

tion here employed CI MS. LC/EI MS proved impractical due to large pressure fluctuations, presumably because of residual solvent left on the belt that was carried to the vaporizer region and the ion source. This is a common problem when a high concentration of water is used in the mobile phase (12). The problem was reduced as much as possible by using a gradient that increased the percentage of organic solvent (acetonitrile) when compounds of interest eluted. Such problems are intensified by improper wetting of the belt due to the high surface tension of aqueous solutions. Operation under CI conditions is the most common practice for LC/MS because it is consistent with the relatively high source pressures encountered in LC/MS (12).

CI Mass Spectra

The PI CI mass spectrum of coumaphos is characterized by the base peak at m/z 363 $(M + H)^+$ and its chlorine isotope peak at m/z 365. This is in contrast to the EI spectrum where ions at m/z 334, 306, 226, 210, 125, 109, and 97 are

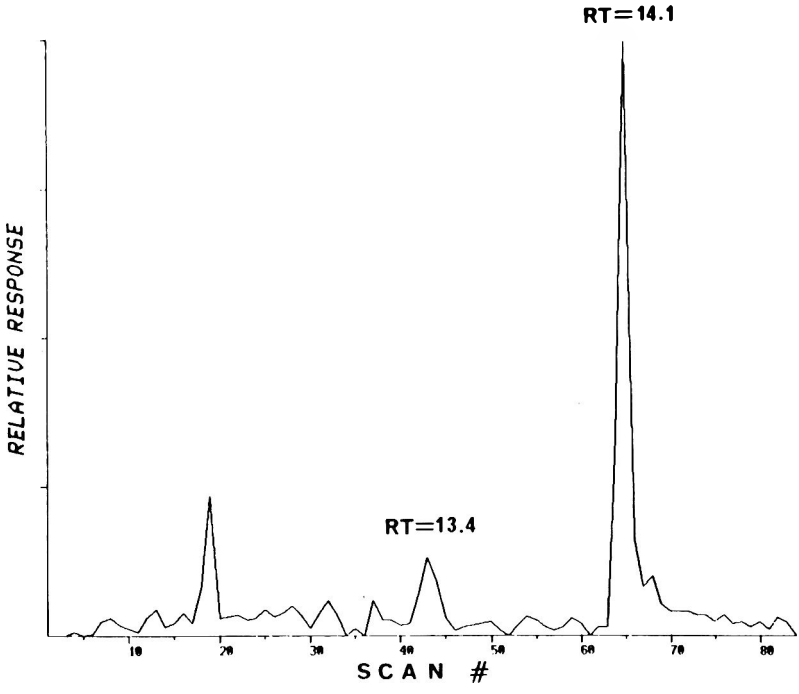

Figure 4. Reconstructed ion chromatogram (5 ions) of coumaphos at 0.1 ppm level in extract of eggs by capillary GC/MS. RT = retention time.

relatively abundant. The ability to acquire NI data simultaneously by the alternative scan technique was used to obtain additional specificity for the confirmation. The negative ion at m/z 225 and the chlorine isotope at m/z 227 result from rearrangement upon electron capture followed by fragmentation, which results in an ion represented by beta cleavage to the ring and replacement of oxygen by sulfur at this position. The ion at m/z 174 corresponds to dissociative electron capture with cleavage beta to the ring and additional loss of chlorine from this moiety. The results are consistent with published spectra (8). Confirmation of coumaphos was based on monitoring 5 ions (positive ions m/z 365, 363 and negative ions m/z 227, 225, and 174). These 5 ions with correct retention time and relative abundance afford a specific confirmation while providing relatively low background response for samples. The GC/MID MS and LC/CI MS mass spectra of coumaphos are shown in Figure 1.

For the oxygen analog, the ions at m/z 347 and 349 of the $(M + H)^+$ cluster together with the negative ions at m/z 153 and 174 were monitored. The ion at m/z 153 corresponds to the moiety (8). Thus a total of 4 ions with correct retention time and relative abundance are used for confirmation. The GC/MID MS and LC/CI MS spectra of the oxygen analog are shown in Figure 2.

GC/MS of Extracts

In this study, extracts of milk and eggs were prepared and spiked at the 0.1, 0.01, and 0.005 ppm levels as described above. For GC introduction, about 4 ng coumaphos was required for injection to obtain reproducible MID spectra.

Figure 3 illustrates the GC/MS results at the 0.1 ppm level for coumaphos and the oxygen analog in extracts from egg and milk matrices, respectively. In general, ion reproducibilities were at or below ±5% relative abundance for the ions monitored. The agreement between spectra of standards and those from extracts was within the limits of the ion reproducibilities (Figures 1–3).

A capillary column chromatogram that represents the sum of the ion currents monitored is shown in Figure 4. Coumaphos and its oxygen analog are separated by about 30 s, with a peak width of about 10 s at the base. Both coumaphos and its oxygen analog are separated from interferences. A practical limitation of about 2 μL injection volume in the splitless mode was not exceeded. Results for coumaphos and its oxygen

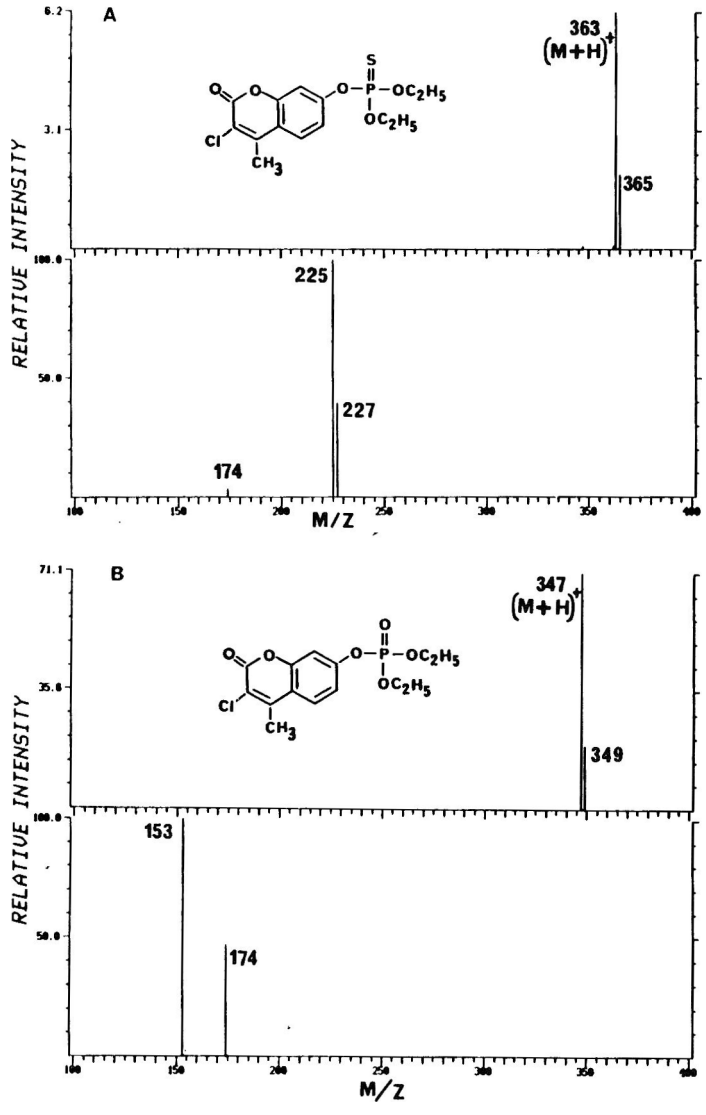

Figure 5. MID (LC/MS) mass spectra at 0.1 ppm level (250°C source, 0.3 torr methane, PI NI). A, coumaphos in milk; B, oxygen analog in eggs. Upper spectrum, PI; lower spectrum, NI.

analog were comparable and consistent with standards at the 0.1 and 0.01 ppm levels. The 0.005 ppm level was subject to large background responses which precluded confirmation. Overall run time was about 15 min.

LC/MS of Extracts

When LC introduction was used, about 20 ng coumaphos per injection was required to obtain reproducible results. Since a split of 20:1 was used, this implies that about 1 ng compound was transferred to the mass spectrometer.

Figure 5 shows the LC/MS results at the 0.1 ppm level for coumaphos and its oxygen analog in extracts from milk and egg matrices, respectively. In general, ion reproducibilities were about 8% relative abundance. The agreement between spectra of standards and those of extracts was within the limit of ion reproducibilities (Figures 1, 2, and 5).

A liquid chromatogram is shown in Figure 6 for the 0.1 ppm level of coumaphos and the oxygen analog in eggs. The recording was made using UV detection. The column effluent was diverted from the mass spectrometer until 1 min before peak elution as indicated by the UV recorder, and was diverted once peak elution was complete as indicated by the mass spectrometer.

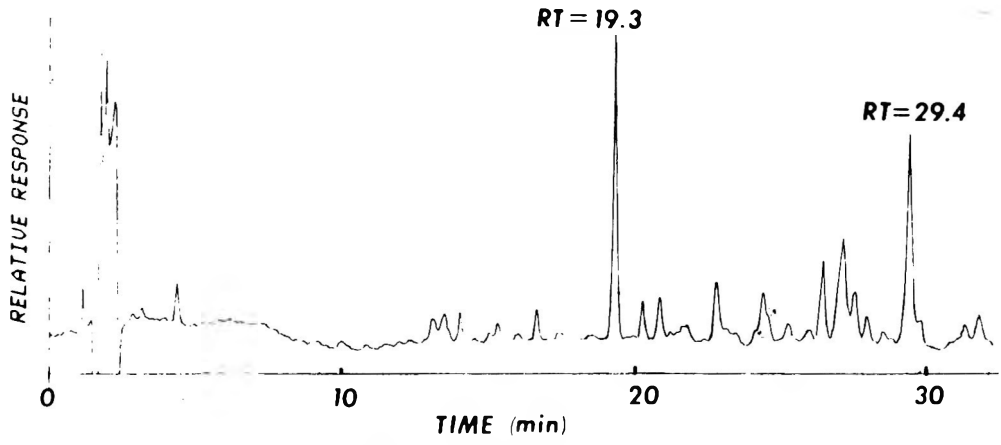

Figure 6. LC/UV chromatogram of 0.1 ppm level of coumaphos and oxygen analog in eggs (1.5 mL/min, gradient water-acetonitrile). RT = retention time.

This procedure kept background buildup and source contamination to a minimum but allowed the mass spectrometer to stabilize before peak elution. The separation of coumaphos from the oxygen analog was about 10 min with a peak width of about 30 s and run time of about 45 min. All 3 levels gave comparable results for the MID mass spectra when LC introduction was used.

Conclusions

The GC/MS and LC/CI MS methods for confirming coumaphos and its oxygen analog in milk and eggs were compared. The efficiency of the LC system and interface appeared greater than the GC system for the actual amount required to obtain a reproducible response (4 ng by GC vs 1 ng by LC). This was probably due to inefficiency in the capillary injector (14).

The reproducibility of ion relative abundances was better in GC/MS than in LC/MS (±5% by GC vs ±8% by LC), but both were acceptable. Pressure fluctuations in the ion source continued to be a major problem in this reverse phase system, and the necessity of a 5% split was a major limitation of LC/MS.

The total analysis time for GC vs LC was favorable (15 vs 45 min). Considerable development time may be required for LC separations combined with MS compared to capillary chromatographic procedures, which are now considered routine. This may in part reflect our greater experience with GC/MS methods, but the greater number of variables in LC/MS is relevant.

Both GC/MS and LC/MS can separate coumaphos and the oxygen analog from matrix effects for the confirmation. However, LC/MS can be used as low as the 0.005 ppm level and therefore is more tolerant of co-extractives than is capillary GC.

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Gas Chromatographic Determination of Mixtures of Captan, Folpet, and Captafol

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A gas chromatographic procedure, using electron capture detection, has been studied and found to be applicable for determining 3 structurally similar fungicides, captan, folpet, and captafol, which are often found in combination in certain foods. Eight liquid stationary phases (AN-600, OV-25, OV-101, OV-210, OV-225, QF-1, SP-2401, and XE-60) were evaluated. A fluorosilicone stationary phase of moderate polarity, SP-2401, was best suited for column stability, response, resolution of captan and folpet, and freedom from on-column decomposition of the 3 fungicides.

The phthalimide fungicides captan, folpet, and captafol are widely used on various fruits and vegetables. Captan and folpet residues are often found in combination during routine sample analysis of certain commodities (e.g., grapes). While all 3 fungicides are readily amenable to electron capture (EC) gas chromatographic (GC) detection (1), captan and folpet are difficult to resolve with conventional packed column chromatography. Stationary phases listed in the *Pesticide Analytical Manual* (PAM) (2) and routinely used for pesticide residue analysis, such as the methyl silicones OV-101 and DC-200 and a mixture of OV-101 and OV-210, will not resolve captan and folpet. On-column degradation of captan and folpet has been observed on OV-101 columns regularly used in the pesticide laboratory for multiresidue screening purposes. Degradation of captan caused by column deterioration has been reported by Carlstrom (3).

GC procedures for captan, folpet, and captafol in various crops were reviewed by Fishbein (4) and Zweig and Sherma (5). Baker and Flaherty (6) reported using 3% XE-60 on Chromosorb W to simultaneously determine residues of captan, folpet, and captafol in several fruits. Captafol in apple wood, leaves, and fruit was determined by Chiba and Northover (7), using 5% QF-1 on Gas-Chrom Q, and by Ngoran et al. (8), using 3% SP-2401 on Supelcoport. Beyer (9) found 3% XE-60 on Gas-Chrom Q to be superior to 3% XE-60 on Chromosorb W(HP) for the determination of captafol in plants and wheat. He noted that conditioning with large amounts of captafol was necessary when using Chromosorb W(HP) support, whereas when using Gas-Chrom Q support, this priming step was unnecessary. A high carrier gas (nitrogen) flow rate of 190 mL/min was used with Gas-Chrom Q. Beyer concluded that the high flow rate coupled with the greater inertness of the support provided a superior column for the determination of captafol.

Büttler and Hörmann (10) encountered difficulties with the changing sensitivity of the EC detector, which they attributed to possible adsorption and/or decomposition during the GC process. Therefore, they developed a residue method for captan, folpet, and captafol based on liquid chromatography and detection by a photoconductivity detector. The GC stationary phases which they investigated are not reported in that paper.

The purpose of the present work was to try to find the optimal stationary phase for reliable GC determination of captan, folpet, and captafol. Several of the stationary phases reported in the literature were evaluated for separation characteristics of captan and folpet. On-column degradation and adsorption were also considered for all 3 fungicides. Packings of various polarity were examined because captan, folpet, and captafol are relatively polar organochlorine compounds.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—Hewlett-Packard 5880A equipped with ^{63}Ni constant current EC detector, Level 4 data station, and Hewlett-Packard 7672A auto sampler. Operating conditions: argon–methane carrier gas (95 + 5), flow rate 30 mL/min; detector temperature 340°C; injection temperature 200°C. Output of detector adjusted to produce ca 50% full-scale deflection (FSD) for 0.3 ng chlorpyrifos.

(b) *GC columns.*—Glass, 6 ft (1.8 m) × 2 mm id, unless otherwise noted. All columns were packed in this laboratory with commercial cus-

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tom-coated packings. Packings and column temperatures were as follows: (*1*) 5% QF-1 on 80–100 mesh Chromosorb W(HP) at 155°C; (*2*) 5% OV-210 on 80–100 mesh Chromosorb W(HP) at 180°C; (*3*) 5% AP-2401 on 100–120 mesh Supelcoport at 175°C (folpet and captan) and 195°C (captafol); (*4*) 3% XE-60 on 80–100 mesh Gas-Chrom Q at 180°C; (*5*) 3% AN-600 on 80–100 mesh Chromosorb W(HP) at 190°C; (*6*) 3% OV-25 on 100–120 mesh Supelcoport (10 ft (3 m) × 2 mm id) at 200°C; (*7*) 3% OV-225 on 100–120 mesh Supelcoport (6 ft (1.8 m) × 4 mm id) at 200°C; (*8*) 5% OV-101 on 80–100 mesh Chromosorb W(HP) at 200°C. Packings in columns 1, 3, 6, 7, and 8 were obtained from Supelco, Inc., Bellefonte, PA 16823. Packings in columns 2 and 5 were obtained from Analabs, Inc., North Haven, CT 06473. Packing in column 4 was obtained from Applied Science Laboratories, Inc., State College, PA 16801.

(c) *Solvents.*—Isooctane (2,2,4-trimethylpentane), distilled in glass (MC&B Reagents, Cincinnati, OH 45212); benzene, distilled-in-glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

(d) *Chlorpyrifos standard solution.*—0.06 μg/mL isooctane.

(e) *Response standard solution.*—For characterizing column suitability. Solution prepared to contain the following compounds (ng/μL isooctane): lindane, 0.02; heptachlor, 0.02; aldrin, 0.04; heptachlor epoxide, 0.04; dieldrin, 0.05; endrin, 0.06; and *p,p'*-DDT, 0.08.

(f) *Captan, folpet, and captafol standard solutions.*—Stock solutions prepared in benzene at ca 100 μg/mL and stored at −7°C when not in use. Working standard solutions for GC diluted from stock solutions with isooctane to ca 0.2 μg/mL for captan and folpet and ca 0.4 μg/mL for captafol.

Results and Discussion

Evaluation of Column Packings

The stationary phases evaluated are chemically classified as phenylmethyldiphenyl silicone (OV-25), cyanopropylmethyl-phenylmethyl silicone (OV-225), cyanoethylmethyl silicone (XE-60 and AN-600), trifluoropropylmethyl silicone (QF-1, OV-210, and SP-2401), and methyl silicone (OV-101). OV-225 is recommended by several chromatography suppliers as a substitute for XE-60. However, it cannot be considered a true equivalent of XE-60 as reflected by the differences in their McReynolds' constants. The McReynolds' values for OV-225 indicate a greater polarity and somewhat different selectivity. AN-600 is reported by Analabs, Inc. (Catalog No. 21) to be a thermally more stable XE-60. OV-210 and SP-2401 offer greater thermal stability than the similar QF-1. Significantly higher column efficiencies are reported for SP-2401 than for OV-210 (11).

Criteria for column evaluation included the following: column stability and efficiency, retention, resolution, and freedom from on-column decomposition and/or adsorption of the 3 fungicides.

Columns prepared with OV-25, OV-225, and QF-1 packings were initially evaluated. Captan and captafol did not elute from the OV-25 column, while folpet chromatography was nondefinitive, resembling excessive decomposition. Captan and folpet could be separated on the OV-225 column. However, this column was unsuitable because of excessive adsorption of the 2 compounds and partial decomposition of folpet to phthalimide on the column. All 3 fungicides separated on the QF-1 column without any on-column decomposition; however, the long retention times required for the satisfactory resolution of captan and folpet and a high baseline noise level due to column bleed made this column impractical to use. Therefore, these 3 columns were eliminated from further evaluation.

The 4 packings that showed the greatest promise for the separation of captan and folpet are listed in Table 1. Retention times relative to captan and response data for the 3 fungicides are also given in Table 1. Baseline resolution of captan and folpet was obtained on the XE-60 and AN-600 columns (Figure 1). However, an elevated baseline, denoting decomposition throughout the length of the column, was observed in the area of the chromatogram preceding the folpet peak on AN-600. The same observation was made in the chromatogram preceding the captan peak on XE-60. Therefore, both columns were eliminated from further investigation. The greatest response of the 3 fungicides was observed using SP-2401 and OV-210 columns. However, on-column degradation was evidenced by elevated baselines preceding elution of the parent compound for all 3 fungicides on OV-210. Therefore, this column was also eliminated from further consideration. The SP-2401 column effectively separated captan and folpet (Figure 2) with no apparent degradation observed for the 3 compounds. This stationary phase best met the desired criteria.

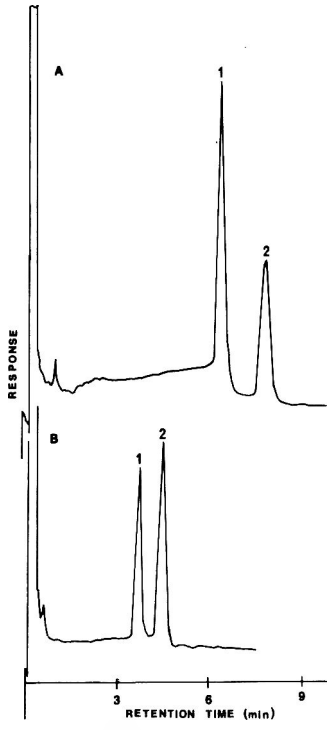

Figure 1. Gas chromatograms of mixture of (1) folpet and (2) captan on: A, 3% AN-600 column at 190°C; B, 3% XE-60 column at 180°C.

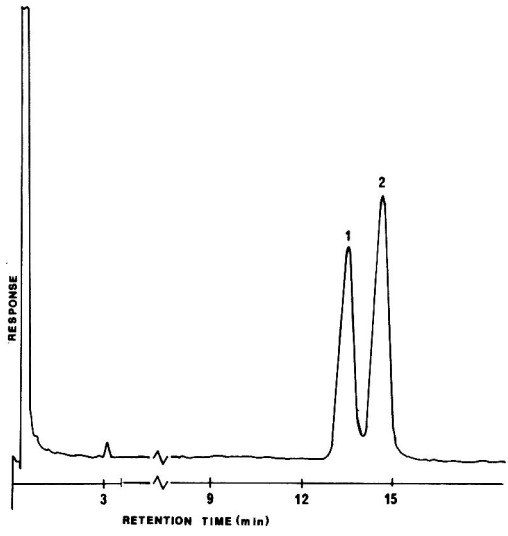

Figure 2. Gas chromatogram of mixture of (1) 0.5 ng folpet and (2) 0.6 ng captan on 5% SP-2401 column at 175°C.

Characterization of the SP-2401 Column

A 5% load of the liquid phase SP-2401 was selected to provide the required inertness of packing necessary when working with compounds that have a tendency to degrade or be adsorbed on lower load packings (12).

The following conditioning procedure (Supelco, Inc., Bellefonte, PA, 1983, private communication) for SP-2401 support gave consistently high efficiency columns. A thoroughly cleaned and silanized 6 ft (1.8 m) glass column was packed with the 5% SP-2401-coated support, using vacuum and a hand vibrator. Silanized glass wool plugs were used at both column inlet and outlet. The column was purged at ambient temperature for at least 30 min with nitrogen gas flow at 40 mL/min to eliminate any trapped air and to further condense the packing. The oven temperature was gradually raised to a maximum

Table 1. GC retention times relative to captan and response data for captan, folpet, and captafol

Column	Column temp., °C	Fungicide	Rel. retent. time	Approx. 50% FSD, ng
3% XE-60	180	folpet	0.82	2.0
		captan	1.0 (4.3 min)	4.6
		captafol	3.63	42.8
3% AN-600	190	folpet	0.81	1.8
		captan	1.0 (7.7 min)	3.9
		captafol	3.24	25.6
5% OV-210	180	folpet	0.92	0.6
		captan	1.0 (10.3 min)	1.0
		captafol	2.56	ND[a]
	185	captafol	(RT = 21 min)[b]	7.7
5% SP-2401	175	folpet	0.92	0.5
		captan	1.0 (14.2 min)	0.5
		captafol	2.56	ND
	195	captafol	(RT = 16 min)[b]	1.0

[a] ND = not determined.

[b] Only captafol was chromatographed at this column temperature. Therefore, retention time (RT) relative to captan was not calculated.

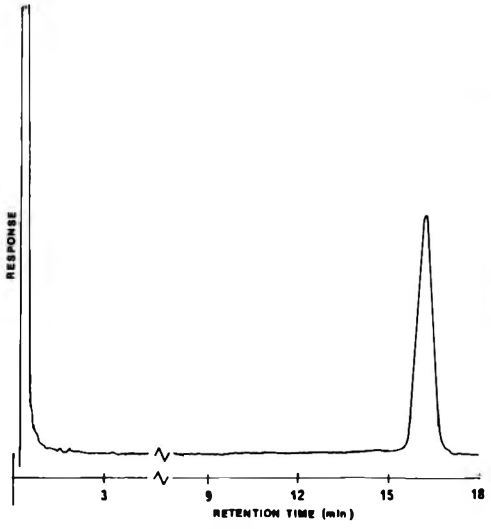

Figure 3. Gas chromatogram of 1.0 ng captafol on 5% SP-2401 column at 195°C.

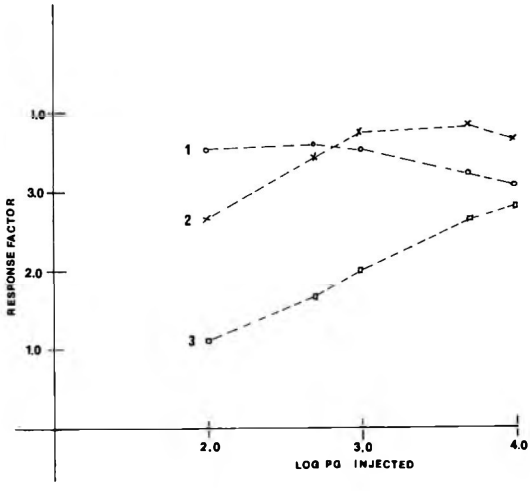

Figure 4. Dynamic response range (peak height/pg vs logarithm pg injected) for (1) folpet and (2) captan at 175°C, and (3) captafol at 195°C on 5% SP-2401 column.

conditioning temperature of 220°C as follows: Oven temperature was raised to 50°C and held for 30 min, then increased to 110°C for 2 h, and finally raised to 220°C and held at that temperature for 3 days. Nitrogen flow rate during column conditioning was maintained at ≥30 mL/min.

The above column conditioning procedure produced a column with an efficiency value of at least 3200 theoretical plates for a 6 ft column. The response standard was chromatographed to evaluate the new column. Column efficiency was determined from computation based on the *p,p'*-DDT peak. The formula $N = 16(x/y)^2$ was used to calculate theoretical plates, where N = total theoretical plates; x = retention time corrected for solvent, mm; and y = width of peak base, mm, measured as the distance at the baseline between lines drawn tangent to the 2 sides of the peak. Endrin was used to evaluate breakdown and adsorption characteristics of the new column. Repetitive injections ($n = 6$) of a standard solution of endrin were made to check reproducibility of peak height size. Good reproducibility was obtained with a coefficient of variation (CV) of 1.3%. The column was further conditioned by injecting nanogram quantities of the 3 fungicides until a constant response was observed (within ±3% or less).

A resolution factor of 1.26 was obtained for captan and folpet at a column temperature of 175°C (Figure 2). The formula $R = 2z/y' + y$ was used to calculate resolution of captan and folpet, where z = the difference between the retention times for 2 components, y' = peak width at baseline of component 1, and y = peak width at baseline of component 2. Captafol quantitations were made at a column temperature of 195°C (Figure 3) because a more reasonable retention time for this compound was obtained at that temperature, i.e., 37 min at 175°C vs 16 min at 195°C.

The dynamic response range for each compound was determined over 3 orders of magnitude (10^2–10^4 pg injected). The response factor (peak height/pg injected) was plotted vs the log pg injected (Figure 4). A straight line parallel to the x-axis indicates a linear dynamic response. The plot shows that the detector used in this study is not linear for captan or captafol over the entire response range tested. However, within smaller segments of this range, the detector displayed adequate linear response for quantitative purposes. Folpet was reasonably linear over the entire range tested.

Captan, folpet, and captafol are known to be particularly susceptible to inconsistent response; therefore, a study was made of the response factor (peak height/pg injected) variability over periods of 1 day to 1 month. As shown in Table 2, the response factors for all 3 compounds were relatively consistent over 1 day. The variability (CV) of response for folpet remained constant over 1 month. However, this variability increased significantly for captan and captafol from 1 day to 1 month. Column temperature does not

Table 2. Response factor variability of captan, folpet, and captafol chromatographed on 5% SP-2401 column

Time	Column temp., °C	Fungicide[a]	No. of injections	Response factor		
				Range	Mean	CV, %
1 month	175	folpet	23	3.32–3.58	3.43	2.6
		captan	23	2.77–3.50	3.15	6.5
		captafol	22	0.72–1.02	0.86	10.3
1 week	195	captafol	5	2.04–2.65	2.36	9.5
1 day	175	folpet	7	3.46–3.73	3.52	2.7
		captan	7	3.66–3.73	3.80	3.1
		captafol	7	1.04–1.12	1.07	2.3
	195	captafol	7	2.25–2.36	2.31	1.7

[a] Amount of each fungicide present in each injection: 0.995 ng folpet, 1.003 ng captan, and 1.998 ng captafol.

appear to have a significant effect on captafol response variability.

Various measures were attempted to improve reproducibility of response. No permanent or significant improvement on response variability was noted by the following manipulations: priming injections of large amounts of the 3 fungicides, complete elimination of glass wool from the column inlet, sample matrix introduction on the column, or the use of a more polar sample solvent such as ethyl acetate. Lowering the detector temperature to 320°C lowered response values but did not improve response variability. Because of inherently greater variability in chromatographic response for the 3 compounds, the response must be monitored frequently with standard injections when samples are analyzed.

Solution Stability

An evaluation of the chemical stability of dilute standard reference solutions of captan, folpet, and captafol was conducted, using solvents suitable for dissolution of the compounds and also compatible with the EC-GC system. Reference stock solutions were prepared at approximately 100 μg/mL benzene. Dilute working standards for GC were prepared in triplicate from the freshly prepared stock solutions in benzene, using isooctane as the diluent and stored as follows: (1) at ambient temperature on the laboratory bench exposed to light; (2) at ambient temperature stored in the dark; and (3) refrigerated at −7°C. These solutions were periodically checked for changes in the concentration and for the appearance of any decomposition products over a period of 3 months. The solutions were injected in triplicate, using an auto sampler, and the gas chromatograms were evaluated. Examination of the chromatograms revealed no chemical instability of captan, folpet, or captafol in isooctane solution under any of the test conditions.

Application to Samples

Extracts of an apple sample fortified with all 3 fungicides and carried through the general method for nonfatty foods in PAM (2) were injected on both the 5% OV-101 column specified in PAM and the 5% SP-2401 column. As can be seen in Figure 5, captan and folpet are insuffi-

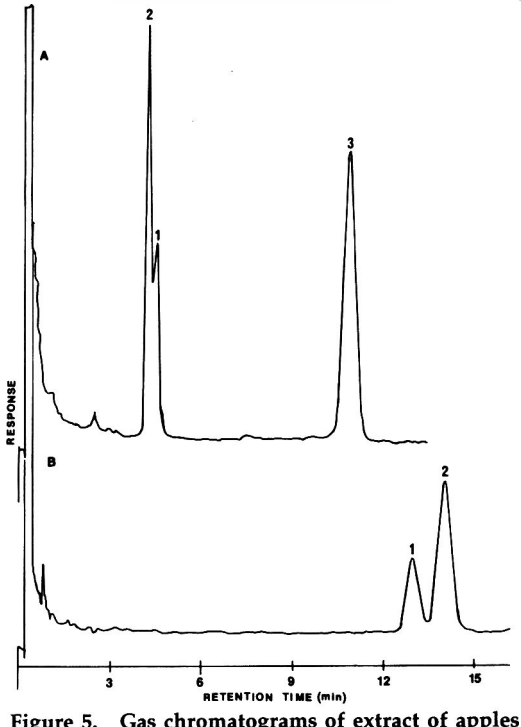

Figure 5. Gas chromatograms of extract of apples fortified with (1) 0.2 ppm folpet, (2) 0.2 ppm captan, and (3) 0.4 ppm captafol on: A, 5% OV-101 column at 200°C; B, 5% SP-2401 column at 175°C. Captofol elutes from 5% SP-2401 column in 37 min at 175°C and is therefore not shown.

ciently resolved on the 5% OV-101 column to permit reliable quantitation. Greater variability of response for individual standards injected concomitantly with sample extracts was obtained on the OV-101 column. For example, the CV of captafol standard responses was 5.3% (n = 7) on the 5% OV-101 column and 1.9% (n = 5) on the SP-2401 column.

Summary

A 5% SP-2401 on 100–120 mesh Supelcoport column prepared and operated under the conditions specified was the most suitable of the several column packings evaluated for the quantitation of captan, folpet, and captafol. This moderately polar material adequately resolves captan and folpet, which are relatively difficult to separate, and thus makes possible the quantitation of residues of these 2 fungicides when present together in foods. The column was superior for all 3 fungicides with regard to stability, efficiency, and absence of on-column compound decomposition, thereby yielding maximum chromatographic response and reproducibility relative to other columns studied. The column has thus far been durable to food sample extracts and will be used in further investigation of the recoveries of captan, folpet, and captafol from various food commodities.

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VITAMINS AND OTHER NUTRIENTS

Semiautomated Fluorometric Method for Determination of Vitamin C in Foods: Collaborative Study

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Continuous flow automation of the microfluorometric procedure compares favorably with the manual method in sensitivity, specificity, and generality, and reduces the cost of routine vitamin C assay. Fifteen samples of 12 different products of ready-to-eat cereals, fruit juices, and infant formula were sent to 6 collaborators; one sample in each category was sent as blind duplicates. The within-laboratory standard deviations for 5 collaborators on the 3 sets of blind duplicates were 1.23, 0.87, and 3.64 mg/100 g, respectively. Overall, the average relative standard deviation between laboratories was 11.1% (range 4.5–16.6%) for the manual method and 4.99% (range 1.5–12.6%) for the semiautomated method. The method has been adopted official first action.

One of the most specific methods for determining total vitamin C in a variety of foods, food ingredients, and food products (1), introduced and studied by Deutsch and Weeks (2, 3), quantitates ascorbic acid and its biologically active oxidation product dehydroascorbic acid. The ascorbic acid present is selectively oxidized to dehydroascorbic acid by using acid-washed Norit. The dehydroascorbic acid is then reacted with *o*-phenylenediamine to produce the fluorescent condensation product 3-(1,2-dihydroxyethyl)furo(3,4-b)quinoxaline-1-one which can then be quantitated by measuring the resulting fluorescence intensity with a fluorometer (excitation 364 nm, emission 440 nm).

The cost of using such methodology for assuring the nutritional quality of food products can be reduced by using continuous flow automation as a means of eliminating various labor-intensive steps. Continuous flow automation of the current AOAC microfluorometric procedure has been reported (4) and compares favorably with the manual method in sensitivity, specificity, and generality. In addition, because it provides greater throughput per unit of analyst's time, it reduces the cost of routine vitamin C assay. Since the ruggedness, specificity, sensitivity, and generality of the manual method had previously been studied collaboratively (3), it was the purpose of the present collaborative study to determine within- and between-laboratory reproducibility of the method when carried out in a semiautomated mode.

Collaborative Study

Fifteen samples of 12 different products from 4 food categories (ready-to-eat [RTE] cereals, fruit juices, milk, and milk-based liquid infant formula) were used. Products which were colored (cranberry, grape, and tomato juices) and which contained sugar were included because they are known to cause difficulty with colorimetric titration and osazone formation methods but not with microfluorometric methodology. Samples were chosen that were of interest to the collaborators participating in the study and covered an approximate vitamin C range of 10–150 mg/100 g sample. Dry samples were ground to pass a 40 mesh sieve, mixed thoroughly, divided, and hermetically sealed in glass containers. Cans of fruit juice and liquid infant formula from a single production lot number were purchased, identifying labels were removed, and the unopened cans were forwarded to the collaborators. Three samples (one each from the cereal, fruit juice, and infant formula groups) were sent to collaborators as blind duplicates. All collaborators were supplied with a copy of the method and instructed to analyze each of the samples once, following the method provided. Estimated vitamin C levels were provided for convenience in making dilutions. Collaborators provided their own primary standards.

This report of the Associate Referee was presented at the 96th Annual International Meeting of the AOAC, Oct. 25–28, 1982, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee D and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67**, March issue.

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Total Vitamin C in Food
Semiautomated Fluorometric Method
First Action

Apparatus

(a) *Automatic analyzer.*—AutoAnalyzer II system equipped with flow scheme shown in Fig. 1 (Technicon Corp.), or equiv.

(b) *Fluorometer.*—Aminco fluorocolorimeter equipped with J4-7413 flow cell and J4-7125 4 watt lamp (American Instrument Co., Silver Spring, MD 20910), or equiv.

(c) *Fluorometer filter.*—Primary 7-60, band pass 70% *T* at 356 nm; secondary Technicon No. 126-0077-01, band pass 40% *T* at 440 nm, or equiv.

(d) *Rainin pipet.*—Model P-5000 (Rainin Instrument Co., Inc., Boston, MA 02135), or equiv.

(e) *Collection funnels.*—DisPo No. F-7501 (Scientific Products, Evanston, IL 60201), or equiv.

(f) *Plastic cups.*—Falcon No. 4020 (Becton, Dickinson and Co., Oxnard, CA 93030), or equiv.

(g) *Osterizer blender.*—Pulsematic 16 (Scientific Products), or equiv.

(h) *Magnetic stirrer.*—Thermolyne (Scientific Products), or equiv.

(i) *Mechanical shaker.*—Wrist-action shaker (Burrell Corp.), or equiv.

(j) *Dispensing pipet.*—Kimax No. 37075-F (Ace Glass Co., Inc.), or equiv.

Reagents

(a) *Brij-35 soln.*—35% in H_2O (Atlas Chemical, Wilmington, DE 19899).

(b) *Extraction solvent.*—4.0% metaphosphoric acid-MeOH (3 + 1, pH 2.1). Prep. fresh weekly.

(c) *Wash soln.*—Add 1.5 mL Brij soln to 1 L extn solv., and filter.

(d) *Dialysate receiving soln.*—1.5 mL Brij soln/L H_2O.

(e) *Sodium acetate solns.*—(*1*) Dissolve 302 g anhyd. sodium acetate in H_2O; dil. to 1 L with H_2O. Add 1.5 mL Brij soln, and filter. (*2*) Prep. soln as in (*1*); adjust pH to that of boric acid soln below (ca 6.9) with HCl.

(f) *Boric acid.*—Dissolve 5 g boric acid in 100 mL sodium acetate soln (*1*). Prep. fresh daily.

(g) *o-Phenylenediamine hydrochloride.*—0.5 mg/mL. Dissolve 50 mg *o*-phenylenediamine HCl (Eastman Kodak Co.) and dil. to 100 mL with H_2O. Prep. fresh daily.

(h) *Acid-washed Norit.*—Add 1 L 10% HCl (1 + 9) to 200 g Norit Neutral (Fisher Scientific Co., carbon, decolorizing, C-170), heat to bp, and filter with vac. Remove cake to large beaker. Add 1 L H_2O, stir, and filter. Repeat washing with H_2O and filtering. Dry overnight at 110–120°.

(i) *Norit slurry.*—Add 20 g acid-washed Norit to extn solv. and dil. to 100 mL. Transfer slurry to 125 mL erlenmeyer equipped with mag. stirrer app. Mix slurry at rate to maintain homogeneity.

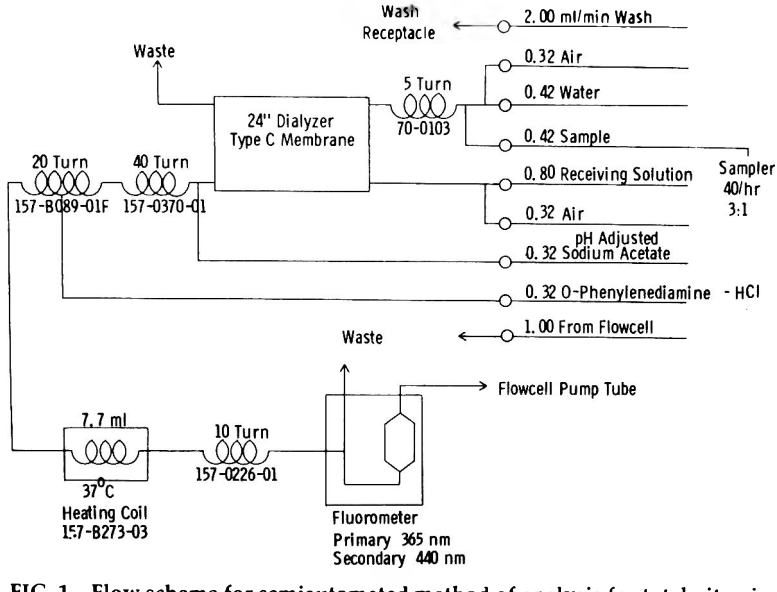

FIG. 1—Flow scheme for semiautomated method of analysis for total vitamin C

Table 1. Collaborative results for semiautomated vitamin C method (mg/100 g)

Lab.	Sample 1—Tomato Juice		Lab.	Sample 2—Wheat RTE Cereal	
1	9.63	12.60	1	116.2[a]	108.8[a]
2	12.80	10.30	2	95.3	94.3
3	9.99	9.76	3	92.2	92.6
4	9.50	9.58	4	92.8	91.8
5	9.92	9.55	5	94.2	92.5
Lab.	Sample 3—Infant Formula (Dry Soy)		Lab.	Sample 4—Orange Juice	
1	117.5	114.1	1	48.7	
2	113.9	105.5	2	48.8	
3	114.9	117.0	3	49.6	
4	110.5	103.8	4	49.3	
5	113.0	114.0	5	47.1	
Lab.	Sample 5—Oat RTE Cereal		Lab.	Sample 6—Grape Juice	
1	64.5		1	30.5	
2	69.7		2	29.6	
3	68.3		3	30.5	
4	69.2		4	30.3	
5	71.5		5	29.1	
Lab.	Sample 7—Corn RTE Cereal		Lab.	Sample 8—Cranberry Juice	
1	88.8[a]		1	34.7	
2	142.9		2	37.6	
3	135.8		3	34.9	
4	142.4		4	34.0	
5	139.0		5	36.9	
Lab.	Sample 9—Apricot Nectar		Lab.	Sample 10—Grapefruit Juice	
1	19.5		1	37.8	
2	19.1		2	30.7	
3	18.8		3	30.3	
4	18.6		4	29.8	
5	18.4		5	29.4	
Lab.	Sample 11—Infant Formula		Lab.	Sample 12—Nonfat Dry Milk	
1	15.1		1	8.7	
2	12.1		2	10.4	
3	11.9		3	9.5	
4	11.2		4	9.1	
5	13.9		5	9.4	

[a] Rejected at 95% confidence level.

(j) *Ascorbic acid standards—(1) 1.0 mg/mL.*—Accurately weigh 100 mg ascorbic acid (Mallinckrodt; store in desiccator) into 100 mL vol. flask, dissolve, and dil. to vol. with extn solv. *(2) 100 μg/mL.*—Pipet 10 mL of above soln into 100 mL vol. flask and dil. to vol. with extn solv. *(3) 2, 10, 25 μg/mL.*—Pipet 2, 10, and 25 mL 100 μg/mL soln into sep. 100 mL vol. flasks, dil. to vol. with extn solv.

Standard Curve

Add 1 mL (Rainin pipet) Norit slurry (0.2 g Norit) to each working std soln (2, 10, 25 μg/mL). Shake and transfer contents of each vol. flask to plastic cups and mechanically shake 10 min. Filter mixt. (Whatman 2V), immediately transfer filtrate from DisPo funnels to AutoAnalyzer sampler, and pump thru instrument. Adjust recorder so 25 μg/mL std gives full scale peak.

(a) *Low moisture samples.*—Grind to pass 40 mesh sieve. Accurately weigh portion of sample (max. wt 5 g) contg ca 1.5 mg ascorbic acid into plastic cup and dispense 100 mL extn solv. Add 1 mL Norit slurry, cap, and shake 10 min. Filter and pump filtrate through AutoAnalyzer. Negligible error is introduced by moisture in sample.

(b) *High moisture samples, fruits and vegetables.*

Table 2. Statistical analysis of collaborative results

Sample		Mean, mg/100 g	SD between labs (rel. %)	
1	tomato juice	10.36	0.64	(6.14)
2	wheat RTE cereal	93.2	1.38	(1.49)
3	infant formula (dry soy)	112.4	4.05	(3.61)
4	orange juice	48.7	0.97	(1.99)
5	oat RTE cereal	68.6	2.59	(3.77)
6	grape juice (purple)	30.0	0.62	(2.07)
7	corn RTE cereal	140.0	3.31	(2.36)
8	cranberry juice	35.6	1.55	(4.35)
9	apricot nectar	18.9	0.43	(2.28)
10	grapefruit juice	31.6	3.50	(11.08)
11	infant formula (milk-based liquid)	12.8	1.61	(12.58)
12	nonfat dry milk	9.4	0.63	(6.70)

Table 3. Vitamin C content (mg/100 g), summary of comparative data

Product Description	No. of samples	Mean	
		Manual	Semiauto.
Cereal and cereal products:			
Oat/corn (colored)	3	82.3	85.2
Wheat/buckwheat	2	214.3	223.0
Oat	2	155.1	148.1
Corn (colored)	3	57.2	61.4
Oat/corn (colored)	1	90.1	85.1
Corn	2	245.0	257.2
Oat/corn (colored)	2	109.6	101.2
Oat/corn	2	77.2	71.8
Oat/corn (colored)	4	99.6	95.6
Corn/wheat	2	92.3	92.6
Oat/corn (colored)	1	155.0	159.6
Corn	2	95.0	95.0
Oat (colored)	1	107.2	100.8
Wheat	2	230.7	237.0
Corn (colored)	3	69.7	65.9
Wheat	2	84.2	85.1
Flour	1	0.0	0.0
Cake mix	2	0.0	0.0
Bread crumbs	1	0.0	0.0
Crackers	1	0.0	0.0
Fruits & vegetables:			
Pears	1	1.0	1.2
Peaches	1	3.3	3.5
Strawberry preserves	1	4.5	4.2
Fruit cocktail	1	1.5	1.5
Corn (whole kernel)	1	2.1	2.1
Tomatoes (stewed)	1	9.2	9.5
Green beans	1	1.1	1.1
Potato buds	1	7.1	6.5
Apple (infant food)	1	18.5	17.1
Pineapple & Banana (infant food)	1	12.6	9.2
Peaches (infant food)	1	19.3	24.7
Tomato juice	1	12.1	12.4
Pineapple juice	1	38.3	38.7
Grape juice	1	44.1	45.6
Orange juice	1	38.3	38.1
Miscellaneous:			
Pizza	9	1.1	1.0
Weiners	1	21.6	16.5
Hamburger	1	0.0	0.0
Pet foods	2	0.0	0.0
Frozen beef dinner	1	0.0	0.0
Frozen chicken dinner	1	0.0	0.0

Table 4. Vitamin C content (mg/100 g) summary of statistical data

Product category	Samples compared	Manual method		Semiautomated method		Corr.	SD of diff.
		Mean	Range	Mean	Range		
Cereals, flour (34 samples),[a] and flour products (5 samples)[b]	39	93.55	0–245.0	93.55	0–257.2	0.9980	5.28
Fruits, vegetables, & fruit-based infant foods[c]	15	14.36	1.1–45.6	14.20	1–44.1	0.993	1.81
Miscellaneous: meats, frozen dinners, pizza, pet foods[d]	15	1.68	0–16.5	2.07	0–21.6	0.999	1.31

[a] Table 1, ref. 4.
[b] p. 650, paragraph 6, ref. 6.
[c] Table 3, ref. 6.
[d] Table 5, ref. 4, p. 650; paragraph 7, ref. 6; and p. 651, paragraph 1, ref. 6.

—Grind sample in blender. Weigh portion of slurry (max. wt 5 g) contg ca 1.5 mg ascorbic acid into 100 mL vol. flask and dil. to vol. with extn solv. Add 1 mL Norit slurry, cap, shake, and transfer contents to cup. Continue as in (**a**).

(**c**) *Dehydroascorbic acid.*—Treat sample as in (**a**) except replace 1 mL Norit slurry with 1 mL extn solv.

(**d**) *Blank determination.*—Replace pH-adjusted sodium acetate line with boric acid soln. Pump stds and samples thru flow scheme to obtain blank values.

Calculation

Plot peak ht of std minus blank vs concn. Obtain sample concn in μg/mL by comparing blank-corrected peak hts for each sample with std curve. No error is introduced by omitting 1 mL diln from Norit slurry if it is omitted in both sample and std calcns. Calc. total vitamin C or dehydroascorbic acid level in original sample as follows:

$$\text{Vitamin C, mg/100 g} = (C/W) \times 10$$

where C = concn, μg/mL, of ascorbic or dehydroascorbic acid from calibration curve; W = sample wt, g; and 10 is a combined factor taking into account 100 mL extract, level reported per 100 g, and conversion of g to mg.

Results and Discussion

Results obtained by the participating laboratories are shown in Table 1. None of the 5 collaborators reported difficulty in analyzing any of the samples submitted. One collaborator reported that when the method is used routinely, a nitrogen bubbler helps to retard degradation of the *o*-phenylenediamine solution, and that shaking the Norit-treated sample by hand 3 times for 30 s over a 10 min time span gives results equivalent to shaking 10 min on a wrist-action shaker. Another collaborator reported that water is routinely used in place of methanol in the extraction solvent. Both collaborators, however, followed the submitted procedure for this study. Collaborator 6 used a modification of the procedure (5), which gave acceptable within-laboratory reproducibility but gave results on all samples significantly lower than these obtained by the other collaborators. Consequently, results obtained by Collaborator 6 were not used in the statistical evaluation of the method.

The within-laboratory standard deviations based on results obtained for blind duplicate Samples 1, 2, and 3 were 1.23, 0.87, and 3.64 mg/100 g, respectively (11.9, 0.93, and 3.2% relative). The between-laboratory standard deviations are shown in Table 2. The standard deviations of the results from the collaborating laboratories were determined and all results lying outside the 95% confidence interval were rejected. The standard deviation was then recalculated. As can be seen, the between-laboratory standard deviation determined by this study is less for the semiautomated procedure than for the manual procedure on which it was based, collaborated in 1967 (3). The average relative standard deviation between laboratories was 11.1% (range 4.5–16.6%) for the manual method, and 4.99% (range 1.5–12.6%) for the semiautomated method.

Recommendation

Based on the historical performance of the manual method (1) on which the semiautomated

method is based (i.e., identical chemical oxidation and condensation reactions and similar extraction procedures), the excellent agreement between the 2 methods when studied independently by 2 different laboratories (4, 6) across a variety of products (see summary, Tables 3 and 4), and the improvement shown by the semiautomated over the manual method for within-laboratory and between-laboratory variability, it is recommended that the semiautomated method for the fluorometric determination of vitamin C in food products be adopted official first action.

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Liquid Chromatography, Microfluorometry, and Dye-Titration Determination of Vitamin C in Fresh Fruit and Vegetables

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The vitamin C content of several fresh fruit and vegetables was determined by a liquid chromatographic (LC) method which gave simultaneous separate values for ascorbic acid and dehydroascorbic acid (DHA) and by the official AOAC methods of microfluorometry and dye-titration. The levels of ascorbic acid obtained by LC and dye-titration were in good agreement, except for a few colored products where it was difficult to determine the end point of the titration. The combined values for ascorbic acid and DHA obtained by LC and microfluorometry were in agreement for most produce, but for about one-third of the samples, the values obtained by microfluorometry were significantly higher.

Fruit and vegetables are the major sources of vitamin C in most diets. The principal biologically active form of vitamin C is L-ascorbic acid; its primary oxidation product, L-dehydroascorbic acid (DHA), is also active, although it is generally present at relatively lower levels (1). There are 2 official AOAC methods for analysis of vitamin C in foods: the dye-titration method (2) which determines ascorbic acid only and is considered to be unreliable because of a range of potentially interfering compounds (3), and the microfluorometric method (4) which is preferable from a nutritional point of view because it produces a value for total vitamin C activity (i.e., ascorbic acid plus DHA) and is less susceptible to interference, but it is a more lengthy method (3).

The technique of liquid chromatography (LC) has been of increasing interest for the analysis of vitamin C. Initial studies analyzed ascorbic acid only, but recent publications (5–8) have reported systems which allow the simultaneous but separate determination of both ascorbic acid and DHA from a single injection. This paper reports a study which compares the levels of vitamin C in 18 fresh fruit and vegetables obtained by using the LC method proposed by Wimalasiri and Wills (8) with those obtained from microfluorometric and dye-titration methods.

Experimental

Fresh fruit and vegetables were purchased from the local market and homogenized in a domestic blender. A sample (15 g) was then blended with either citric acid or metaphosphoric acid solution (50 mL) and diluted to volume (100 or 250 mL) with extracting solution. The resulting solution was filtered through paper (Whatman 541) and the filtrate was used for analysis.

The LC method was used exactly as described by Wimalasiri and Wills (8). Produce was extracted with 3% citric acid solution, filtered through paper, and further purified by passage through a membrane/ultrafilter cell (Diaflo ultrafilter, Amicon Corp.) and a short disposable column containing μBondapak C_{18} (C_{18} Sep-Pak, Waters Associates). An aliquot (20 μL) was analyzed for ascorbic acid and DHA on a μBondapak/carbohydrate column (Waters Associates) (30 cm × 4 mm id) installed in a Waters liquid chromatograph (Model ALC/GPC 244) equipped with a 41-mPa pump and U6K injector. Column effluents were monitored by 2 UV detectors set at 254 nm (Waters Model 440) and 214 nm (Waters Model M441) for estimation of ascorbic acid and DHA, respectively. The mobile phase was acetonitrile–water (70 + 30 v/v) containing 0.01M ammonium dihydrogen phosphate (pH 4.3) at 2 mL/min.

The microfluorometric method as described in AOAC (4) involves extraction with 3% metaphosphoric acid solution, and oxidation of ascorbic acid with Norit to DHA. An aliquot (2 mL) was reacted with *O*-phenylenediamine solution to give a fluorescent quinoxaline derivative which was assayed in a fluorometer. The dye-titration method as described in AOAC (2) involved extraction with 3% metaphosphoric acid solution which was titrated with 2,6-dichloroindophenol to a distinct rose-pink end point.

The initial study was done on 18 types of fruit and vegetables. For reproducibility, 6 samples of 5 types were analyzed by each method.

Results and Discussion

Table 1 gives the vitamin C content of 18 fruit and vegetables analyzed by LC, fluorometry, and dye-titration. Comparison of total vitamin C

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Table 1. Vitamin C content (mg/100 g) of fresh fruit and vegetables determined by LC, fluorometry, and dye-titration [a]

Produce	LC			Fluorometry	Dye-titration
	Ascorbic acid	DHA	Total		
Banana	9.6	3.1	12.7	11.9	10.4
Broccoli	66.0	2.3	68.3	80.5	67.8
Brussels sprouts	54.0	8.0	60.0	65.0	53.8
Cabbage	41.0	3.0	44.0	45.0	43.1
Capsicum	142.0	3.2	145.2	145.8	143.7
Cauliflower	47.1	2.6	49.7	54.0	46.5
Celery	5.2	2.0	7.2	7.8	7.0
Cumcumber	7.4	1.6	9.0	8.5	7.6
Kiwifruit	92.3	4.7	97.0	101.2	95.7
Lemon	49.7	0	49.7	52.0	49.0
Lime	32.0	0	32.0	33.5	30.2
Oranges	54.0	0	54.0	56.2	50.9
Parsley	130.6	4.1	134.7	140.5	131.0
Potatoes	10.2	2.0	12.2	12.7	10.8
Rockmelon	62.0	0	62.0	63.6	60.2
Spinach	44.0	8.2	52.2	56.0	46.3
Strawberries	46.0	5.3	51.3	50.7	(56.0)[b]
Tomatoes	10.2	0	10.2	10.9	(13.5)[b]

[a] Each value is the mean of 2 samples.
[b] End point of titration difficult to determine because of colored extract.

activity by LC and fluorometric methods show that, while the values for 10 products differed by less than 2 mg/100 g, 6 products differed by more than 3 mg/100 g; broccoli generated values differing by about 12 mg/100 g. In all products with substantial variations, the value obtained by fluorometry was greater than that obtained by LC. This may result from an underestimation of the level of DHA by LC, but this seems unlikely because the recovery of DHA added to homogenized produce was 93.2% ± 2.0 (n = 6). Alternatively, the fluorometric method may be overestimating vitamin C. While the fluorometric method is generally claimed to be less susceptible to interfering substances, there are reports (9, 10) of enhanced values due to interference. The products giving the greatest differences were mostly dark green vegetables; it is possible that the interference is pigment-related. The results obtained in this study differ from those obtained by Floridi et al. (11) who reported substantially lower values for 5 of 9 fruit and vegetables by the fluorometric method compared with an LC method which gave a single value for total vitamin C activity by estimation after reduction of all forms of ascorbic acid. It would seem that their LC values were inflated by some interfering substance.

Estimations of ascorbic acid by LC and dye-titration are comparable; examination of the data in Table 1 shows relatively good agreement for most produce: values obtained for 12 products differ by less than 2 mg/100 g. Disregarding the values for strawberries and tomato where the colored extracts gave unreliable dye-titration results, average values for the 16 products were 52.9 mg/100 g by LC and 53.4 mg/100 g by dye-titration; the largest differences in any pair of readings were about 3 mg/100 g for kiwifruit and oranges. Fresh fruit and vegetables do not contain substantial amounts of compounds which interfere with the dye-titration method.

Data in Table 2 show that values obtained by each method were reproducible when the same food homogenate was analyzed 6 times; standard deviations were about 1 mg/100 g for the 3 methods. A recovery trial with ascorbic acid added to each food sample produced a mean recovery of 96.3% with no significant difference (P = 0.05) in the recovery between the 3 analytical methods. Although the absolute values in Table 2 differ from the values for the corresponding produce in Table 1 because of biological variation among batches of produce, they show similar effects due to methods; all values for ascorbic acid by LC and dye-titration are similar while values for total vitamin C activity by fluorometry for broccoli, brussels sprouts, and parsley are higher than those obtained by LC and values for cabbage and capsicum are similar.

As a general method for analysis of total vitamin C activity of fresh fruit and vegetables, LC gives a more reliable estimate than the fluorometric method, although for about half of the

Table 2. Reproducibility of vitamin C estimated by LC, fluorometry, and dye-titration (mg/100 g ± SD)[a]

Produce	LC			Fluorometry	Dye-titration
	Ascorbic acid	DHA	Total		
Broccoli	57.2 ± 1.0	5.3 ± 0.8	62.5 ± 0.9	74.0 ± 0.7	56.5 ± 0.7
Brussels sprouts	66.8 ± 1.1	3.3 ± 0.9	70.1 ± 0.9	77.6 ± 0.9	67.3 ± 1.5
Cabbage	41.7 ± 1.0	3.1 ± 0.5	44.8 ± 0.7	43.9 ± 1.4	42.8 ± 0.8
Capsicum	150.0 ± 1.4	2.1 ± 0.5	152.1 ± 0.9	151.6 ± 1.6	149.0 ± 1.2
Parsley	130.3 ± 0.9	5.6 ± 0.4	135.8 ± 0.7	140.8 ± 0.5	130.8 ± 0.6

[a] Each value is the mean of 6 samples.

produce examined, both methods were similar. However, where both methods are suitable, the fluorometric method may be preferable because it is less time consuming and less expensive in terms of chemicals and other consumable items. The use of Sep-Pak cartridges was necessary to purify the extracts (8) but adds significantly to the time and cost of the analysis. A simpler method of purification is desirable. Many workers analyze only for ascorbic acid; while this would simplify the LC method, the presence of DHA in most fruit and vegetables would result in an underestimation of vitamin C activity. If only ascorbic acid values were required, the dye-titration method would give adequate results for horticultural produce.

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Differential Pulse Polarographic Determination of Iodine in Foods and Nutritional Products

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A differential pulse polarographic procedure is described for the analysis of iodine in foods and nutritional products. Samples with iodine concentrations ranging from 5000 to 0.2 ppm have been successfully analyzed using this procedure. Precision averaged between 2 and 10% relative to the iodine level measured. Recoveries of added iodine ranged from 89 to 108% with external standards, and 97–100% by an analyte additions technique. Samples analyzed include dried almonds, whey protein concentrate, nonfat dried milk, sea kelp, vitamin-mineral nutritional supplements, diet meal replacement products, dried green beans, dried mushrooms, and wheat germ.

Iodine is an essential micronutrient found in the body, primarily in the thyroid hormones thyroxin and triiodothyronine. These hormones act in the control of the basal metabolic rate. The U.S. Recommended Daily Allowance (USRDA) (1) of iodine for adults and children over the age of 4 years is 150 μg/day. Published estimates (2) indicate that the mean dietary intake in the United States ranges from 600 to 825 μg/day for adults.

The reliable determination of low levels of iodine has traditionally posed a significant problem for the analyst, and serves as the basis for the several investigations which appear in the literature. The techniques generally used include ion specific electrode (3–12), X-ray fluorescence (7, 12), neutron activation (7, 11, 13–19), spectrophotometric (7, 11, 14, 16, 19–21), gas chromatographic (7, 22–25), volumetric (7, 26), and differential pulse polarographic (7, 27–29) methods.

Colorimetric and titrimetric procedures are both subject to masking interferences, and the detection of the titrimetric end point can be impaired by sample turbidity. X-ray fluorescence and neutron activation techniques are cost-prohibitive due to the instrumentation involved and, in the case of neutron activation analysis, a chemical separation must be performed before irradiation to prevent the interference of chlorine and bromine compounds. Ion-specific electrode procedures normally require that the iodine be present as a single ionic specie, and these methods are sensitive to interferences from sulfhydryl compounds. Physical interferences also pose a problem because lipid materials can build up around the sensor, which causes drift and changing response. The gas chromatographic determination of iodine relies on the derivatization of the iodine to an iodoketone, which is then measured using an electron capture detector. This procedure is lengthy and is prone to losses through incomplete derivatization and extraction. Internal standards are normally used, but cannot always reflect the actual losses that occur. This procedure is also sensitive to varying protein and lipid levels that may be present in the sample. Differential pulse polarography (DPP) suffers from some of the same drawbacks that plague other techniques; the DPP technique is specie-specific, and minerals may interfere in the analysis. These problems are generally easily remedied by the addition of various reagents to mask other electroactive compounds and convert the iodine to a single ionic form. The apparent versatility and flexibility of the polarographic procedure of Holak and Shostak (27) was the basis for our investigation into the use of DPP in the analysis of food and nutritional products.

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Experimental

Apparatus

The analytical system used for the polarographic analyses consisted of an EG&G Princeton Applied Research Model 384 polarographic analyzer, with a Model 303 mercury electrode using an Ag/AgCl reference electrode, and a Houston Instrument X-Y recorder. Operating conditions were:

initial potential	−0.8 V (vs Ag/AgCl)
final potential	−1.3 V
drop size	large
scan increment	4 mV
scan rate	4 mV/s
pulse height	100 mV
argon purge time	240 s
drop time	1.0 s

Samples were prepared for analysis using Vycor or nickel crucibles and a laboratory box furnace.

Reagents

Sodium carbonate, bromine, potassium iodide, calcium phosphate, and sodium sulfite were all J. T. Baker Chemical Co. reagent grade chemicals purchased from a local laboratory supply house.

Procedure

A portion of representative ground sample, 1–10 g depending on iodine content, is weighed directly into a suitable crucible. Approximately 4 ± 0.1 g anhydrous sodium carbonate is mixed into the sample. Another 4 ± 0.1 g portion of sodium carbonate is added to the sample to overlay the sample mixture. Samples that have an appreciable moisture content (>10%) require preliminary drying before alkaline fusion. The sample is placed in a cool furnace and the temperature is increased to 700°C and held for 30–60 min. After cooling, the residue is dispersed in 15–20 mL hot deionized water and filtered through glass fiber paper (GF/A) into a 50 mL volumetric flask. One mL saturated bromine water is added to oxidize any iodide present to the iodate form. Approximately 20 ± 1 mg sodium sulfite is added to remove excess bromine. The sample solution when diluted to volume with deionized water is ready for polarographic analysis using the parameters outlined in *Apparatus*. A 10 mL aliquot of the sample is quantitatively transferred to the polarographic cell and deaerated with argon. Significant amounts of zinc interfere with the determination of iodine due to an overlapping peak at −1.25 V. This interference can be effectively masked by the addition of 2 mL 1% dibasic calcium phosphate solution to the polarographic cell before analysis.

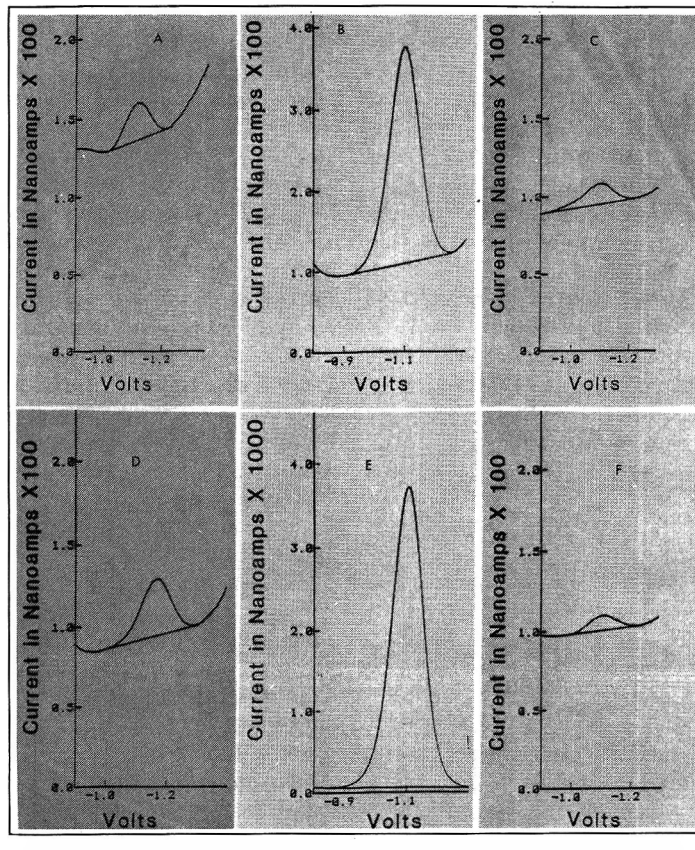

Figure 1. Polarograms of iodine in (A) dried apple granules: 10 g sample yielded 24 nA peak corresponding to 0.26 μg I/g; (B) vitamin-mineral nutritional supplement: 10 g sample yielded 271 nA peak equivalent to 7.5 μg I/g; (C) dried almonds: 10 g sample yielded 13 nA peak equivalent to approximately 0.11 μg I/g; (D) diet meal replacement product: 5 g sample yielded 36 nA peak equivalent to 1.05 μg I/g; (E) Pacific sea kelp: 0.5 g sample diluted to 100 mL yielded 4440 nA peak equivalent to 1630 μg I/g; (F) wheat germ: 10 g sample yielded 5 nA peak, iodine present at 0.1 μg/g.

Table 1. Typical precision of DPP procedure for iodine in foods and nutritional products

Sample	Iodine present, μg/g	Iodine found, μg/g	RSD, %	N
Vitamin/mineral tab.	38 (label)	42.3	4.8	16
Chewable vit./min. tab.	5.7 (label)	5.9	4.6	16
Sea kelp (Pacific)	1500 (minimum)	1630	1.7	11
Diet meal replacement	0.9 (label)	1.05	9.0	16
Nonfat dried milk	unknown	3.1	10.5	10
Dried green beans	unknown	0.70	5.7	5
Vitamin-protein fortified food bar	33.3 (label)	24.7	17.7	5

The peak due to iodate reduction to iodide is found at a potential between −1.10 and −1.15 V. Peak heights drawn from baseline tangents typically yielded a response of 1.0 μA for a solution containing iodine at the 2 μg/mL level. The method of analyte additions was used for all determinations.

Results and Discussion

The analysis of food products for iodine requires a technique that is sufficiently sensitive to accurately measure less than 10 ppm (w/w) iodine. Practical limits were 10 g for sample size and 50 mL for final solution volume. Increasing the sample size required use of a 100 mL crucible for sample ashing. We found that quantitative transfer to a 50 mL flask was extremely difficult. The limiting current amplitude for quantitation was determined to be 20 nA. A peak with an amplitude of approximately 20 nA could be generated by a solution containing 0.04 μg iodine/mL, which would be equivalent to a sample concentration of about 0.2 ppm iodine. Figure 1A shows a polarogram of 0.2 ppm iodine in dried apple granules. Iodine can be detected but not accurately determined to approximately 0.1 μg/g.

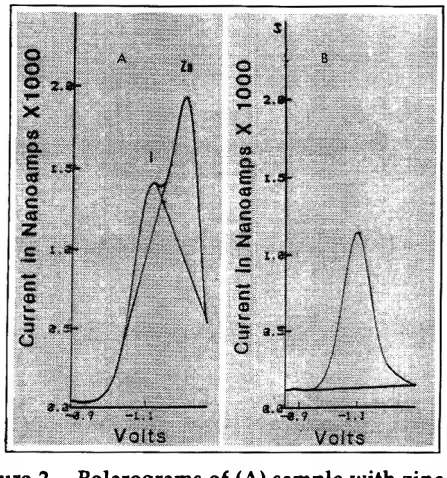

Figure 2. Polarograms of (A) sample with zinc interference; (B) with zinc interference removed by addition of 2 mL 1% dibasic calcium phosphate solution.

The linearity, precision, and accuracy of the procedure were evaluated. The DPP system gave linear response to iodine from 0.04 (20 nA) to 25 μg/mL (125 × 100 nA). The optimal amplitude range was 50–5000 nA which was obtained by controlling the sample size. The precision of the procedure as measured by the relative standard deviation (RSD) averaged 7.7% for the 7 different sample types replicated (Table 1). Recoveries of added iodine from 6 different representative sample types varied from 89 to 108% when an external calibration curve was prepared using matrix-matched iodide standards. To compensate for matrix effects, the method of analyte additions was used. Recoveries using analyte additions calibration ranged from 97 to 100% of added iodine for the same set of samples carried through the procedure. Potassium iodide was used as the iodine source for recovery studies after experiments with iodate and periodate indicated no difference in recovery due to differing ionic form. Samples were spiked at levels from 25 to 100% of their natural level before being analyzed.

Zinc exhibited a significant interference due to an overlapping peak at −1.25 V. Depending on the zinc concentration, this interference could completely obscure the iodine peak (Figure 2A). This was not observed in tableted nutritional supplements containing zinc (>10 mg/g) and approximately 40 μg iodine/g; orthophosphates present in these samples effectively masked the zinc interference. Addition of a 50-fold excess of phosphate relative to the zinc concentration adequately removed the zinc interference (Figure 2B). The mechanism by which the phosphate masks the zinc interference is not precisely known at this time.

A variety of processed foods and nutritional

Table 2. DPP determination of total iodine in processed foods and nutritional products

Sample	Iodine, μg/g	N
Sea kelp—Pacific, powdered	1629	11
Sea kelp—Pacific, granular	1694	4
Sea kelp—Atlantic, powdered	5100	3
Nonfat dry milk	3.1	10
Whey protein concentrate	7.1	2
Soy protein isolate	<0.1	1
Soy flour	<0.1	1
Spirulina	<0.1	1
Mushrooms, dried	<0.1	1
Peas, dried	0.1 approx.	1
Green beans, dried	0.70	5
Celery stalk powder, dried	0.60	1
Bell pepper powder, dried	0.26	2
Wheat germ	0.1 approx.	2
Almonds	0.1 approx.	2
Apple granules, dried	0.26	2
Carrot powder, dried	0.33	2
Chopped onions, dried	0.1 approx.	2
Beet powder, dried	0.78	1
Raisins, dried	<0.1	1
Vegetable soup mix, dried	0.5	2
Chicken soup mix, dried	0.48	2
Onion soup mix, dried	<0.1	2
Apple slices, dried	<0.1	1
Carrot slices, dried	0.40	2
Whole grain, cereal wheat	<0.1	1
Flaked tuna, canned	0.24	2
Vitamin-mineral nutritional supplement		
A	42.3	16
B	33.4	2
C	57.2	3
D	5.9	16
E	19.3	2
Diet meal replacement product		
A	0.74	1
B	2.2	2
C	1.1	2
D	1.0	16
E	1.1	2
F	1.2	2
G	2.3	2
Vitamin-protein fortified food bar	33.3	5
Milk chocolate candy bar	0.8	1

products were analyzed for iodine content by using the described DPP procedure; the results are shown in Table 2. Representative polarograms are shown in Figure 1.

Conclusions

This procedure has been found to be accurate and precise for the measurement of low levels of iodine in many different food types. The analytical technique employed is easily available to most testing laboratories with respect to capital investment costs. The procedure is fairly rapid and can measure levels as low as 0.2 μg I/g, using a 10 g sample and a 50 mL dilution. Measurements at levels approaching 0.1 ppm are possible but precision suffers due to the extremely small current being measured (approximately 20 nA).

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FLAVORS

Acid Methanolysis and Gas Chromatographic Determination of Brominated Vegetable Oils in Soft Drinks

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A gas chromatographic method has been developed for determination of brominated vegetable oils in citrus-flavored soft drinks. Oils were extracted from the drinks with ethyl ether and subjected to acid-catalyzed methanolysis. The resulting brominated methyl esters (dibromostearate, tetrabromostearate, and hexabromostearate) were separated and quantitated as single peaks on a 3% OV-3 column. Chromatography columns were stable for about 3 months of daily use; then the first 15 cm of column packing material was replaced or a new column was prepared. A number of citrus soft drinks were analyzed and contained 3.5-3.9 mg brominated oil/10 fl. oz. Recoveries from spiked samples after organic extraction and methanolysis were 94.5-105%.

Brominated vegetable oils (BVOs) are widely used as dispersing agents for flavoring oils in citrus-based soft drinks. Methods for their determination or characterization have been developed using gas chromatography (1-4), X-ray fluorescence spectrometry (2, 5) specific ion electrodes (6), and titration (6, 7). Of these, only gas chromatography is capable of differentiating and identifying individual BVOs. The method of Conacher (3), adopted official final action by AOAC, uses sodium methoxide treatment of the oils to simultaneously esterify and form derivatives which were amenable to gas chromatography. Lowry and Tinsley (4) improved on this method by making minor changes to the procedure and in the reagents used. In spite of these improvements, the method is still somewhat tedious and uses corrosive reagents, thus a suitable alternative would be preferred. With this in mind, we carried out further investigations on the direct gas chromatographic determination of BVOs after acid methanolysis as described earlier (1) and found that, with certain precautions, reproducible results may be readily attained. Details of this work are described herein.

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METHOD

Reagents

(a) *Solvents.*—Ethyl ether, hexane, and methanol; dry over anhydrous sodium sulfate.

(b) *Acid–methanol solution (2% v/v).*—Dissolve 4 mL reagent grade sulfuric acid in dry methanol and dilute to 200 mL with methanol in volumetric flask.

(c) *Brominated standards.*—Prepare methyl 9,10-dibromostearate (DBS) from methyl oleate; methyl 9,10,12,13-tetrabromostearate (TBS) from methyl linoleate; and methyl 9,10,12,13,15,16-hexabromostearate (HBS) from methyl linolenate in the same manner as previously described (3). Alternatively, they may be purchased from Nu-Chek-Prep, Inc., Elysian, MN.

(d) *DBS standard solution.*—Dissolve 75 mg DBS in ethyl ether in 50 mL volumetric flask and dilute to volume with ethyl ether.

(e) *TBS standard solution.*—Prepare as in (d) above.

(f) *HBS standard solution.*—Prepare as in (d) above.

(g) *Methyl pentadecanoate (MPD) internal standard solution.*—Dissolve 75 mg MPD (Sigma Chemical Co.) in ethyl ether in 100 mL volumetric flask and dilute to volume with ethyl ether.

(h) *Brominated vegetable oils.*—Brominated olive, sesame, and cottonseed oils were obtained (previously) from Abbott Laboratories (North Chicago, IL); brominated soybean, corn, and canbra oils were prepared in the laboratory according to Conacher et al. (1); a commercial brominated oil, Akwilox 133, now in current use, was obtained from Swift Specialty Chemicals Co. Ltd (Chicago, IL). Dissolve 75 mg oil in ethyl ether in 50 mL volumetric flask and dilute to volume with ethyl ether.

Apparatus

(a) *Transesterification flask (T-flask).*—125 mL conical flask with 24/40 joint, with neck elongated and constricted to 8–10 mm id.

(b) *Gas chromatograph.*—Varian 2100 with flame ionization detector, fitted with 6 ft × 2 mm (id) glass column packed with 3% OV-3 on 80–100 mesh Chromosorb W HP. Temperature program, 150–280°C at 10°/min. Helium carrier gas flow, 30 mL/min. Injector and detector temperature, 275°C. Peak areas were measured with Autolab IV computing integrator.

Procedure

(a) *Acid methanolysis.*—Pipet 1.0 mL (1.5 mg) DBS, 2.0 mL (3.0 mg) TBS, 4.0 mL (6.0 mg) HBS standard solutions, and 3.0 mL (4.5 mg) of each BVO solution into separate T-flasks. Add 2.0 mL (1.5 mg) MPD internal standard solution to each flask and evaporate solvent under nitrogen stream at 40°C. To each flask, add 25 mL acid-methanol reagent and 6 mL dry hexane. Reflux 1 h, cool, and add 40 mL water. Mix thoroughly and add more water to bring hexane layer within constricted neck of flask. Remove hexane by pipet into 15 mL glass vial. Add another 4 mL hexane to flask, mix, and remove organic layer by pipet to same vial. Add 1 g anhydrous sodium sulfate to vial, mix, and filter mixture through Whatman No. 1 paper into clean vial. Evaporate hexane under nitrogen stream and dissolve residue in 0.5 mL ethyl ether for gas chromatographic analysis. Inject 2 μL for analysis.

(b) *Extraction of soft drinks.*—Add 2 mL MPD solution to 280 mL (10 fl. oz) soft drink. Saturate mixture with sodium chloride; then extract with three 75 mL portions of ethyl ether. Combine ether extracts and wash first with two 30 mL portions of 2N NaOH and then with 30 mL 2N HCl. Finally, wash with three 30 mL portions of water. Dry over anhydrous sodium sulfate, filter, and evaporate extract to small volume. Quantitatively transfer solution to clean T-flask, evaporate solvent under nitrogen stream, and continue with transesterification as described in preceding paragraph.

(c) *Determination of BVO.*—Inject, in duplicate, DBS, TBS, HBS, all BVO standards, and samples prepared above and obtain peak areas (PA) from integrator printout. Calculate average weight response factor (RF) for DBS, TBS, and HBS:

$$RF(DBS, TBS, or HBS) = [mg(DBS, TBS, or HBS) \times PA(MPD)]/[mg(MPD) \times PA(DBS, TBS, or HBS)]$$

For brominated vegetable oils, calculate weight of DBS, TBS, or HBS as follows:

$$mg\ DBS, TBS, or HBS = [PA(DBS, TBS, or HBS) \times RF(DBS, TBS, or HBS) \times mg(MPD)]/PA(MPD)$$

To identify BVO, calculate ratio of TBS to DBS as follows:

$$R_{T:D} = mg(TBS)/mg(DBS)$$

Total brominated ester content (B) for each oil is calculated by:

$$B = [mg(DBS) + mg(TBS) + mg(HBS)]/mg(BVO)$$

After identification of the BVO using the $R_{T:D}$ from the drink samples, calculate the quantity of BVO present by the equation:

$$mg(BVO) = [mg(DBS) + mg(TBS) + mg(HBS)]/B$$

Results and Discussion

Figure 1 shows typical chromatographic results obtained via acid methanolysis. It can be seen that the reaction yields single peaks for DBS, TBS, and HBS; a somewhat complicated picture is observed with sodium methoxide treatment which, although it produces a single peak for DBS, yields mixtures of unknown compounds for TBS and HBS as shown in Figure 2. In addition, acid methanolysis yields a single peak for HBS, unlike the sodium methoxide treatment which gave a complex mixture resulting in a broad, rising baseline with no consistency from one analysis to the next. The ability to detect HBS by acid methanolysis provides useful information in characterizing unknown BVOs. For example, of the oils which could conceivably be used in soft drinks, only brominated soybean and canbra oils would contain this acid to any significant extent. Typical response factors and equivalent carbon numbers for the brominated esters are as follows: DBS: 1.9, 23.6; TBS: 3.2, 28.9; HBS: 7.1, 36.4, respectively.

Quantitatively, the acid methanolysis provides essentially the same results as the sodium methoxide procedure. Table 1 shows the fatty acid composition of 6 BVOs analyzed after acid methanolysis. These compare very well with results obtained earlier by using sodium methoxide (1, 2).

To maintain the quality of the chromatographic separations, certain precautions must be taken. It was reported earlier that although direct gas chromatographic analysis of the brominated methyl stearates initially provided single peaks, deterioration of the peaks occurred shortly

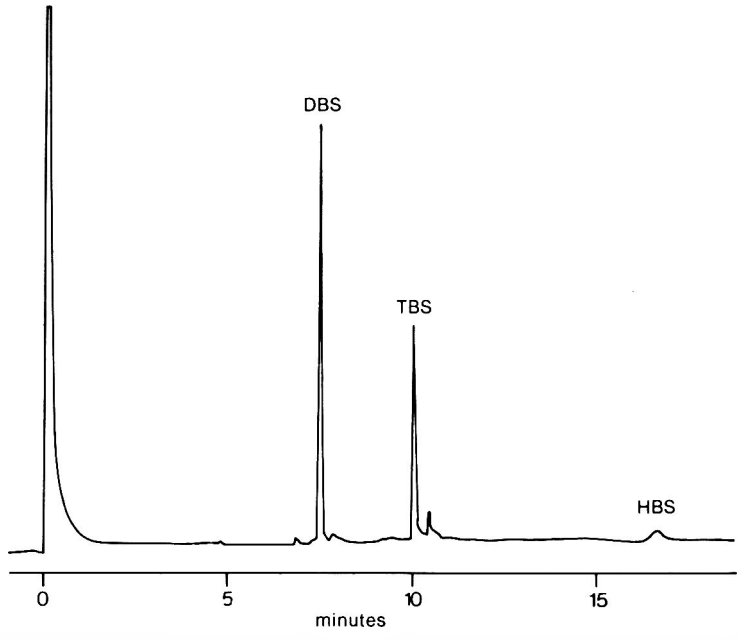

Figure 1. Typical chromatogram obtained from an injected mixture of DBS (6 μg), TBS (12 μg), and HBS (24 μg) standards carried through acid methanolysis and chromatography as described in text.

thereafter, preventing any useful conclusions to be drawn. We have found that by using on-column injection with glass columns, the peak quality was maintained for 3 months or more with little change in response factors for all 3 brominated esters. HBS was the most difficult to quantitate reproducibly due to its long retention time and poor detector sensitivity. However, by conditioning the column with injections of HBS before beginning a sample run, a single reproducible peak for HBS is obtained. Normally, for convenience, a mixture of all 3 brominated methyl stearates (about 700 ng each) is injected daily before analyses are commenced. It was also observed that over the 3 month period, decomposition products gradually accumulated at the head of the column, as was found earlier (1). When the first 10–15 cm of column packing material was replaced, chromatographic integrity immediately improved and the column was useful for another 1 or 2 months of daily use. In practice, after 3–4 months, we normally packed a new column.

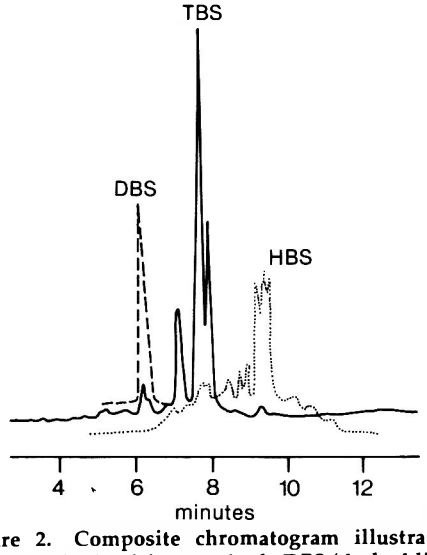

Figure 2. Composite chromatogram illustrating products obtained for standards DBS (dashed line), TBS (solid line), and HBS (dotted line) via sodium methoxide reaction as described in ref. 3. Chromatography conditions as described in text.

The method was applied to the identification and quantitation of BVOs in citrus-flavored soft drinks. Figure 3 shows typical chromatographic results for several soft drinks. Because interferences were observed for the C16:0 and C18:0 peaks (see Figure 3), the TBS/DBS ratios were used to identify the oils, and quantitation was based on the brominated esters only. Possible interferences from other constituents in the soft drinks amounted to only about 1–2% of the permitted level, which is far more than adequate for regulatory purposes. Table 2 lists results obtained for 5 BVO-containing citrus-flavored soft

Table 1. Fatty acid composition of BVOs via acid-catalyzed methanolysis

Brominated Oil	Amt used, mg	Amt recd, mg	Rec. %	Fatty acid composition (area %)						Ratio TBS/DBS
				16:0	18:0[a]	Di-Br	Tetra-Br	Hexa-Br		
Olive[b]	4.56	4.42	97.0	8.1	4.8	78.3	8.8	—		0.11
Sesame[b]	4.81	5.06	105.2	5.6	4.5	40.8	49.2	—		1.21
Cottonseed[b]	4.87	4.67	95.9	14.4	1.4	18.3	65.8	—		3.60
Corn[c]	4.01	4.15	103.4	5.6	0.9	19.5	74.0	—		3.79
Soybean[c]	4.67	4.92	105.3	6.3	2.3	22.0	64.6	4.8		2.94
Canbra[c]	4.85	5.05	104.2	1.8	0.8	56.0	27.3	14.0		0.49
Akwilox 133	4.81	4.26	88.5	6.8	3.1	50.0	39.6	0.4[d]		0.79

[a] Small amounts of unsaturated C18 esters are included in 18:0.
[b] Commercial sample.
[c] Lab-brominated.
[d] Peak area was estimated.

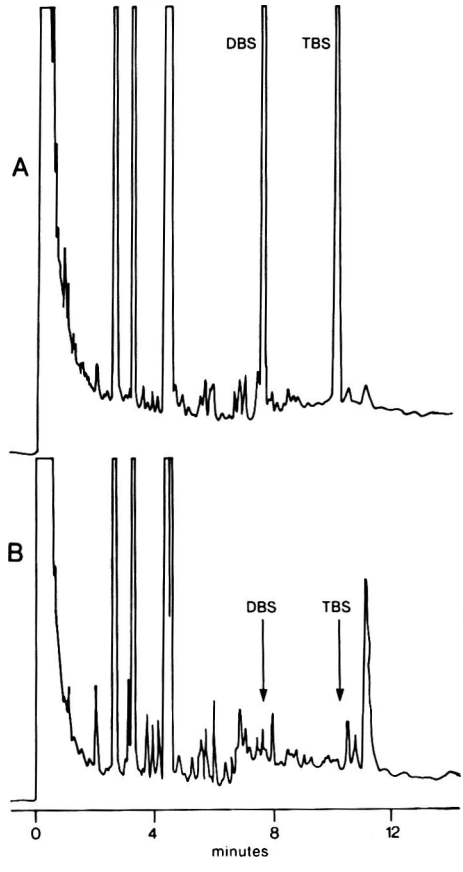

Figure 3. Chromatograms of citrus soft drink samples: A, sample containing BVO; B, sample with no BVO added (arrows indicate retention times of DBS and TBS). Chromatography conditions as described in text; equivalent of 1.12 mL of soft drink injected.

drinks. The ratios, except for one sample (C) were similar to that observed for Akwilox 133, which is known to be used in citrus soft drink preparations. Sample C appears to contain a BVO mixture from a source different from the commercial preparation obtained here.

It can be observed from Table 1 that the TBS/DBS ratio of Akwilox 133 does not correspond to that of any of the individual BVOs. It is possible that the commercial material is a blend of oils, with one of the components being either brominated canbra or soybean oil as indicated by the presence of a small quantity of HBS. However, a more likely reason is that the oils used to prepare Akwilox 133 were partially hydrogenated, thus altering the ratios of the di- and tetra-brominated esters. Holmer and Aaes-Jorgensen (8) reported a fatty acid composition for

Table 2. BVO in citrus-flavored drinks (10 fl. oz)

Sample	DBS, mg	TBS,mg	Ratio TBS/DBS	BVO found, mg[a]
A	1.46	1.24	0.84	3.69
B	1.65	1.11	0.67	3.77
C	0.59	1.27	2.15	—
D	1.49	1.11	0.74	3.56
E	1.52	1.15	0.76	3.65
F	1.78	1.06	0.60	3.88
G[b]	none	none	—	—
Akwilox 133	—	—	0.79[c]	—

[a] Calculated as commercial BVO (Akwilox 133).
[b] Contained no BVO and was used as blank for recovery studies.
[c] From Table 1.

partially hydrogenated soybean oil that included C18:1 (40.8%) and C18:2 (34.8%) which, if brominated, would yield a TBS/DBS ratio of about 1.15, a value close to the 0.79 ratio obtained by us for Akwilox 133. Further evidence for partial hydrogenation was obtained by analyzing Akwilox 133 on a 5% DEGS column in an attempt to separate any *erythro*-dibromostearate (indicative of hydrogenation) from threo-dibromostearate. These 2 isomers are not separated on the 3% OV-3 column. Results indeed did show the presence of a peak corresponding to the *erythro*-isomer where none was present in laboratory brominated soybean oil that was not hydrogenated.

Recovery studies on the soft drinks were carried out by spiking a known quantity of the Akwilox 133 BVO preparation in Sample G (chromatogram shown in Figure 3B), in triplicate, and then extracting and esterifying as described under the method. The average recovery was 100.8% (range 94.5–105.0). The quantities of BVOs found in 10 fl. oz samples of the soft drinks are listed in Table 2. These are within the permitted 15 ppm (4.2 mg/10 fl. oz) levels set by Canadian Food and Drug Regulations.

Conclusions

The described method was found to be simpler than the official final action method (3) because it does not require metallic sodium, benzene, or anhydrous conditions. With certain precautions, consistent single peaks are obtained for DBS, TBS, and HBS, enabling the characterization and quantitation of BVOs in citrus-flavored soft drinks.

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PESTICIDE FORMULATIONS

Liquid Chromatographic Assay for Dalapon Grasskiller Products: Collaborative Study

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As a replacement for the present mercury reagent titration assay for dalapon grasskiller products, a liquid chromatographic method has been developed and collaboratively studied. The method separates the active ingredient, 2,2-dichloropropionic acid, from its related products by paired ion chromatography with UV detection at 214 nm. Statistical analysis of the data indicates that a single assay from one laboratory should not differ by more than 2.2% from the average assay of many laboratories, at the 95% confidence level, for products containing about 75% 2,2-dichloropropionic acid equivalent as the magnesium and/or sodium salt. The method has been adopted official first action.

The present AOAC method for assay of dalapon grasskiller (1) requires a mercury salt reagent (consuming about 10 g mercury oxide per assay) which is difficult to dispose of properly. In addition, the present method assumes a 91% complete reaction between dalapon (active ingredient 2,2-dichloropropionic acid) and the mercury reagent (2), but differences from this value have been observed, depending on the batch of reagent prepared (J. Strope, Dow Chemical USA, Midland, MI; and M. White, Dow Chemical Ltd, Norfolk, UK).

We developed and collaboratively studied a liquid chromatographic (LC) assay for dalapon grasskiller that eliminates the problem of mercury waste disposal and requires the use of a standard.

Collaborative Study

Each collaborator was given 4 samples to assay. Samples 1 and 2 were the sodium salt of dalapon. Samples 3 and 4 were the sodium/magnesium salt of dalapon known as DOWPON M grasskiller (trademark of the Dow Chemical Co.). These 2 forms of the product are representative of almost all dalapon grasskiller sold worldwide. The samples were thus arranged to follow Youden's procedure (3) for closely matched pairs. Each collaborator was also given the chemicals necessary to prepare the eluant, a dalapon acid standard, the chromatographic columns required, and a copy of the procedure. Collaborators were instructed to prepare the standard and each sample in duplicate, and were instructed to check one standard against the other before beginning the analysis of samples.

Dalapon (Magnesium and/or Sodium Salt) in Pesticide Formulations Liquid Chromatographic Method First Action

Method Performance

Dalapon acid equiv. 75% (S_x = 1.1; S_o = 0.63)

Principle

2,2-Dichloropropionate is sepd from related compds on reverse phase LC column, using paired-ion eluant, and detected by UV spectrophtry.

Apparatus

(**a**) *Liquid chromatograph.*—Fitted with 5000 psig pressure gage, 214 nm UV detector, line filter in eluant reservoir, 20 μL loop-type injection valve, and strip chart recorder. Computing integrator optional. Operating conditions: temp., 20–30°; eluant flow rate, 160 mL/h; detector sensitivity, 0.25 AUFS.

(**b**) *Liquid chromatographic columns.*—No. 316 stainless steel, 50 × 4.6 (id) mm Co:Pell ODS pellicular guard column (No. 6561-404, Whatman Inc., 9 Bridewell Pl, Clifton, NJ 07014, or equivalent); 100 × 8 (id) mm C-18, 10 μm radial compression main column with RCM-100 column holder (Waters Associates, Maple St, Milford, MA 01757, or equivalent (such as Whatman Partisil 10-25-ODS-3)). Columns slowly degrade in use. Replace when 30% loss of retention occurs and repack first 5 mm of guard column. Radial compression column recommended gives lower

This report of the Associate Referee, T. S. Stevens, was presented at the 96th Annual International Meeting of the AOAC, Oct. 25-28, 1982, 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 the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67**, March issue.

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back-pressure and longer column life than std 316 stainless steel columns.

Reagents

(a) *Eluant.*—Dilute mixt. of 200 mL CH_3CN (UV grade), 1.6 mL *n*-octyl amine (Eastman P7588 or equivalent), and 2.4 g $(NH_4)_2HPO_4$ (Baker 1-0784 or equivalent) to 1 L with H_2O (0.4 μm filtered.). Adjust pH of eluant to 7.0 with H_3PO_4. Eluant may be recycled if reservoir is mag. stirred and no more than 200 injections are made per L eluant.

(b) *Dalapon std soln.*—130 mg/50 mL. Accurately weigh ca 130 mg dalapon acid ref. (99+% isomer pure; available from Dow Chemical USA, Sample Coordinator, 9001 Building, Midland, MI 48640) into 50 mL vol. flask, add H_2O to mark, and shake. Do not use std soln after 24 h. Det. % H_2O in dalapon acid ref. std by Karl Fisher titrn. Labeled purity of ref. std. is on anhyd. basis and must be appropriately reduced according to H_2O content.

Preparation of Sample

Accurately weigh sample contg ca 130 mg dalapon acid equiv. into 50 mL beaker. Quant. transfer to 50 mL vol. flask with H_2O. Fill to mark with H_2O and shake. Do not use sample soln after 24 h. Dalapon acid and salts are hygroscopic; protect from moisture in air by storing in well sealed bottle. Duplicate sample and std prepn is recommended.

Determination

Inject std soln, sample soln, and then std soln. Calc. results as

$$\% \text{ Dalapon acid equiv.} = (R/R') \times (W'/W) \times P$$

where R and R' = average peak ht or peak area of dalapon peak for sample and std solns, resp.; W and W' = mg sample and std, resp.; and P = % purity of std. Note: Initial system stability may be poor. Before injecting sample soln, repeat injection of std soln to confirm system stability. Periodically confirm linearity by analyzing stds contg 100, 130, and 160 mg 99+% dalapon acid/50 mL H_2O. Flush injection loop with ≥1 mL sample or std soln before operating injection valve.

Discussion

The liquid chromatographic separation mode used in this procedure is paired-ion chromatography (PIC) using *n*-octylamine as the PIC reagent (4). The procedure separates 2,2-dichloropropionic acid from its related products (see Figure 1). Results were linear by peak area measurement for samples containing from 0.05 to 0.17 g 2,2-dichloropropionic acid/50 mL. A

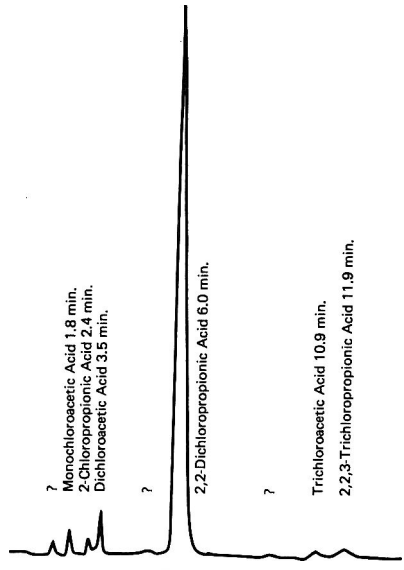

Figure 1. Example chromatogram of 2,2-dichloropropionic acid and related products.

gradual deviation from linearity was noted above 0.17 g/50 mL with only 96% recovery observed for samples containing 0.28 g 2,2-dichloropropionic acid/50 mL.

The reverse phase columns slowly degraded in use, as evidenced by a decreased retention time for 2,2-dichloropropionic acid and decreased resolution of 2,2,3-trichloropropionic acid from trichloroacetic acid. This degradation was thought to be caused by the slow dissolution of the silica-based column packing by the pH 7 buffered eluant. Use of the radial compression column minimized this problem because compression of the column packing prevented formation of a void space at the inlet of the column. In continuous use, the recommended Radial-PAC column needed replacement every 3–5 weeks.

Conventional ¼ in. od 316 stainless steel columns filled with reverse phase packing (such as Whatman Partisil 10-25-ODS-3) gave chromatograms similar to that in Figure 1, with longer or shorter retention times, depending on the carbon loading (%C_{18}, %C_8, or %C_3) on the specific column packing. The pressure drop was also higher across these conventional columns.

Results

Table 1 shows the assay data for 6 collaborators and 4 samples with each sample run in duplicate. Data in Table 1 were subjected to the series of statistical computations outlined in the AOAC

Table 1. LC assay of dalapon grasskiller products: results in duplicate as % 2,2-dichloropropionic acid equivalent

	Sample 1		Sample 2		Sample 3		Sample 4	
Coll.	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
1	74.96	74.25	70.21	70.16	75.39	74.51	74.51	73.77
2	74.96	75.26	70.26	72.36	77.79	77.36	75.84	76.61
3	75.61	74.02	72.02	71.37	74.88	74.30	72.87	73.61
4	75.27	75.32	71.97	69.78	74.57	75.70	75.14	73.68
5	73.89	74.65	70.13	70.43	72.81	73.76	73.18	73.85
6	75.14	75.37	72.48	71.81	75.55	75.18	75.18	74.93

Table 2. Ranking of data in Table 1, results of Dixon test for outlying individual results, and results of homogeneity test for interlaboratory and interreplicate comparisons

Item	Sample 1	Sample 2	Sample 3	Sample 4	All
Coll.	Rank				
1	2	1	3	3	9
2	4	4	6	6	20
3	3	5	2	1	11
4	6	3	4	4	17
5	1	2	1	2	6
6	5	6	5	5	21
Test result	Dixon Test				
Highest	0.04	0.23	0.52	0.39	
Lowest	0.33	0.05	0.30	0.09	
Test result	Homogeneity Test				
Interlaboratory range	0.13	0.21	0.38	0.28	—
Interreplicate range	—	—	—	—	0.12

Table 3. Analysis of variance of the data

Source of variation	Mean sq. Samples 1 & 2	Mean sq. Samples 3 & 4	Ratio of higher to lower mean sq.
Random (S_r^2)	0.3993	0.3976	1.00
Systematic (S_b^2)	0.2686	1.2111	4.51

statistical manual (3); Table 2 shows the evaluation of laboratory ranking. The criterion for rejecting the results of a laboratory due to a high or low total rank with a 1 in 20 probability of a wrong decision with 4 samples and 6 laboratories is a ranking lower than 5 or higher than 23 (Table B of reference 3). Thus, no data need to be rejected by total rank.

Table 2 also shows the results of the Dixon test for outlying individual results for each sample. The criterion for rejecting an outlying result with a 1 in 20 probability of a wrong decision is a value exceeding 0.56 (Table C.1 of reference 3). Thus, no data need to be rejected by the Dixon test.

Table 2 shows the results of the homogeneity test for variation among laboratories and between replicates. The criterion for testing homogeneity of variation from ranges with a 1 in 20 probability of a wrong decision is a test result greater than 0.42 for 6 laboratories and 4 ranges (interlaboratory comparison) and a test result greater than about 0.18 for 24 ranges (interreplicate comparison) (see Table D.1 of reference 3). No results need to be rejected for lack of homogeneity of variation among laboratories or between replicates.

Table 3 lists the analysis of variance according to Youden's procedure for closely matched pairs (3). Almost no difference occurred between the random mean squares (S_r^2) for the 2 sets of samples. Although the difference between the systematic mean squares (S_b^2) for the 2 sets was larger, it was not significant according to the

Table 4. Equipment used by collaborators

Pump	Injector	Column	Detector	Integrator
LDC Constametric	Rheodyne 7125, 20 μL loop	Waters Radial-Pac C-18	LDC-1203, 214 nm	measured peak ht
Varian 5000	Valco,[a] 25 μL loop	Waters Radial-Pac C-18	Perkin-Elmer LC-55-B, 214 nm	Perkin-Elmer Sigma-10
Waters M-6000	Waters U6K, 25 μL syringe	Waters Radial-Pac C-18	Waters 450, 214 nm	measured peak ht
Perkin-Elmer Series-3	Perkin-Elmer Model 420 10 μL loop	Waters Radial-Pac C-18	Perkin-Elmer LC-55-B 214 nm	Perkin-Elmer Sigma-10
Perkin-Elmer Series-3	not noted	Whatman Partisil 10-25-ODS-2	Perkin-Elmer LC-55, 214 nm	measured peak ht
Waters M-6000A	Rheodyne 7125, 20 μL loop[b]	Waters Radial-Pac C-18	Perkin-Elmer LC-55, 214 nm	measured peak ht

[a] With Varian Model 8050 autosampler.
[b] Also tried Waters U6K syringe injector for Samples 1 & 2 with similar results (75.32, 76.06 and 72.47, 71.51, respectively) as obtained with 20 μL loop injector.

F-test (Table E of reference 3). According to this test, the ratio of the higher mean square to the lower mean square (for 5 degrees of freedom for each mean square) must exceed 5.0 for the difference to be significant, with a 1 in 20 chance of a wrong decision. Therefore, the S_r^2 and S_b^2 data in Table 3 were averaged, resulting in an S_r^2 value of 0.3984 for all samples and an S_b^2 value of 0.7398 for all samples.

The interlaboratory standard deviation (S_d) was calculated according to the formula $S_d = \sqrt{S_r^2 + S_b^2}$. Interlaboratory SD ($S_d$) was 1.1; interlaboratory CV was 1.4; SD for one laboratory (S_1) was 0.63; CV for one laboratory was 0.85; SD of systematic error between laboratories (S_b) was 0.86; and CV for systematic error between laboratories was 1.2. The data indicate that this method is adequate for the regulation of labeled assay of dalapon grasskiller products composed of the magnesium and/or sodium salts of dalapon acid (2,2-dichloropropionic acid) and having an acid equivalent assay of about 75%. The data indicate that a single assay in one laboratory should not differ by more than 2.2% from the average assay obtained by many laboratories, at the 95% confidence level.

Collaborators' Comments

Most collaborators noted that 3–6 injections of standard were needed before stable response factors were observed. Several collaborators noted that standards and samples were hygroscopic, but did not think that this property affected their results. One collaborator diluted the samples with eluant instead of water to facilitate the use of an auto-injector (the eluant has a lower viscosity than water). Several collaborators thought the method would be improved if it used an internal standard (we searched for an internal standard during method development without success).

Equipment Used

Collaborators used a wide variety of equipment as outlined in Table 4. One collaborator used a conventional reverse phase column and reported no difficulties. Two collaborators used or experimented with a Waters U6K syringe injection valve without apparent problems. The use of such a valve in an external standardization method such as this requires exact displacement of the syringe plunger for precise results.

Recommendation

The Associate Referee recommends that this method be adopted official first action.

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SUGARS AND SUGAR PRODUCTS

Titratable Acidity in Corn Syrup: Collaborative Study

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Six sets of blind duplicate samples of commercial corn syrups, including high fructose corn syrups, were distributed to 9 collaborating laboratories. Samples were titrated with alkali, according to the AOAC method, to a phenolphthalein "faint pink" end point as well as being assayed by a modified method specifying a larger sample, more dilute alkali, and electrometric end points at pH 6.0, 7.0, and 8.3. Repeatabilities and reproducibilities were about as poor in the AOAC method as in the modified method at pH 8.3, but improved considerably when the modified electrometric end point method was carried out to pH 6.0. The method calling for electrometric titratable acidity at pH 6.0 end point was adopted official first action, and the present method, 31.217, was deleted.

The official AOAC method for determining titratable acidity as percent hydrochloric acid in corn syrup (1) calls for dilution of a 50 g sample to 200 mL, followed by titration with 0.1N sodium hydroxide to a phenolphthalein faint pink (pH 8.3) end point. Early in 1982, the Society of Soft Drink Technologists (2) suggested a guideline specification for titratable acidity of high fructose corn syrups (HFCS) in which measurement would be carried out electrometrically to pH 6.0. This prompted a re-evaluation of early work by the Analytical Procedures Committee of the Corn Refiners Association, who advocated an electrometric titration to pH 7.0 (3), and recommended a collaborative study covering several alternative approaches. The collaborative study and its results are the subject of the present report.

HFCS products are all ion-exchanged as a part of their manufacturing process; therefore, their titratable acidities are quite low, on the order of 0.0030% HCl, whereas conventional acid-converted and dual (acid-enzyme or enzyme-enzyme) conversion corn syrups tend to have acidities as much as 10 times higher. In addition, the actual titers will be smaller for either sample type when measured to an end point of pH 6.0 instead of higher pH values or phenolphthalein end points.

The potentially adverse effect on precision of low acidities and small titers should be compensated, at least for HFCS samples, by the sinusoidal slope of their titration curves, typical of unbuffered systems. Figure 1 shows one such curve in contrast with a conventional starch hydrolysate syrup curve: The former rises vertically with an inflection point at pH 6.0; the latter rises less steeply and in a gradual manner. Both level to a constant pH shortly after attaining pH 8.0. The steeper the titration curve around the chosen end point, the less sensitive the analysis should be to end point variability. The opposite should be true for measurements carried out over the nearly horizontal pH 8 region.

To evaluate the above effects separately and in combination, we decided to compare the official AOAC method, using a visual phenolphthalein end point, with an electrometric end point method at pH 6.0, 7.0, 8.3 and using twice as much sample (100 g) and a more dilute alkali (0.005N).

Collaborative Study

Twelve blind duplicate samples described below were sent to 9 participating laboratories.

1 & 4: 55 DE dual conversion starch hydrolysate syrup (DE = dextrose equivalent or percent reducing sugars dry basis expressed as dextrose (4))
2 & 12: 42% fructose syrup (HFCS-42), producer A
3 & 6: 55% fructose syrup (HFCS-55)
5 & 11: 42% fructose syrup (HFCS-42), producer B

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The recommendations of the Associate Referee were approved by the General Referee and Committee D and were adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67**, March issue.
[1] Present address: CPC International, Inc., Corn Products, Moffett Technical Center, Box 345, Argo, IL 60501.

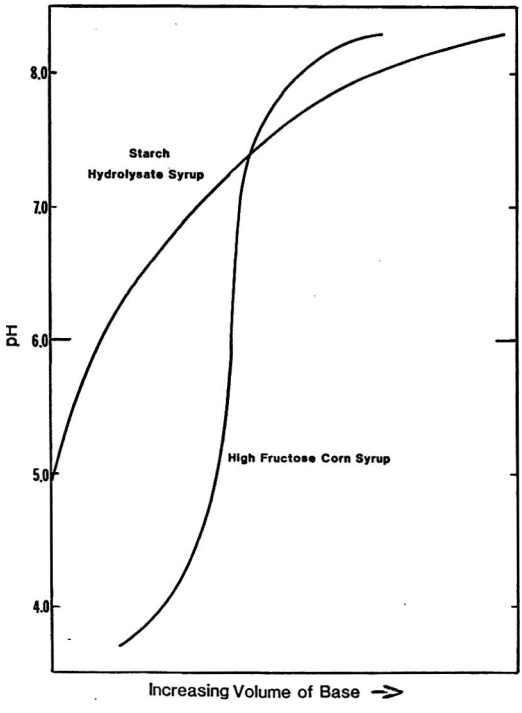

Figure 1. Typical titration curves of corn syrups.

7 & 9: 60 DE dual conversion starch hydrolysate syrup

8 & 10: 42 DE straight acid conversion starch hydrolysate syrup

Collaborators were asked to perform one analysis per sample following the proposed electrometric procedure to pH 6.0, 7.0, and 8.3, respectively, and following the official AOAC procedure to visual phenolphthalein indicator end point. In addition, 4 collaborators agreed to perform complete titration curves on each sample, again following the electrometric procedure.

Acidity (Titratable) in Corn Syrup First Action

Method Performance

Ranges, 12 Duplicate Blind Samples, at pH End Point

Statistic	*6.0*	*7.0*	*8.3*
Mean[a]	2.5-4.5	3.1-10.7	9.3-28.6
Reprod.	0.17-0.37	0.40-0.60	0.84-3.1
CV, %	6.7-9.1	4.9-12.9	5.7-30.5
Repeat.	0.07-0.22	0.13-0.29	0.4-1.02
CV %	1.4-6.3	1.2-7.4	1.6-10.0

[a] % HCl × 10^{-3}

pH = 6.0 end point is recommended based on superior performance despite lower titers.

Apparatus

pH meter.—Capable of reading to 0.01 pH unit. Calibrate instrument just before use against pH 6.0 buffer (see **50.010** or use commercial buffer soln).

Determination

Weigh 100 g sample, dissolve in 150 mL H_2O, immerse electrode(s) of pH meter, stir gently, and titr. with 0.05N NaOH to pH 6.0. Calc. acidity as % HCl.

Results and Recommendations

Titration curves adhered to the expected pattern in Figure 1, sinusoidal curves resulting for the HFCS samples and smooth monotonic curves resulting for the hydrolysate sample.

Acidity data were evaluated according to Steiner (5). Three laboratories submitted incomplete reports, that is, for the AOAC method, hereafter referred to as the phenolphthalein end point (phthln) method, Laboratory A gave no data, and Laboratories C and G, respectively, failed to report on Samples 2 and 11. Thus a statistical evaluation of the phthln method could be performed either on 6 laboratories and 6 samples or on 8 laboratories and 4 samples. The former seemed preferable because it would maintain more data in the evaluation, but, more important, it would avoid dropping 2 of 3 HFCS sample types, which would imbalance the comparison among methods and samples.

From 3 to 4 outlying laboratories ranked high or low of the 9 laboratories in each electrometric method, and 2 outlying laboratories of the 6 laboratories in the phthln method, as follows:

pH 6.0: Laboratories A, H, I
pH 7.0: Laboratories A, C, H, I
pH 8.3: Laboratories A, B, D, I
phthln: Laboratories D, F

When these laboratories were eliminated, the analysis was reduced to 6 laboratories and 6 samples for the pH 6.0 method, 5 laboratories and 6 samples for the pH 7.0 and pH 8.3 methods, and 4 laboratories and 6 samples for the phthln method. Under these circumstances, Dixon outliers were disregarded to maintain as many data as possible.

Analysis of variance was performed before and after removal of outlying laboratories, in the expectation that any statistical trend would become clear after the largest portion of systematic error was eliminated. All information is presented in Tables 1-4. Data in the tables are ex-

Table 1. Titratable acidities as % HCl × 10^{-3} in corn syrups, using 0.05N NaOH to pH 6.0 end point

	Sample											
Lab.	1	4	2	12	3	6	5	11	7	9	8	10
A[a]	5.3	4.7	2.9	2.9	2.7	3.3	3.3	3.3	4.7	5.3	4.0	4.9
B	4.2	4.2	2.5	2.6	2.4	2.5	3.2	3.7	3.9	4.0	3.5	3.4
C	5.1	5.0	2.8	2.8	2.3	2.4	3.0	3.4	4.9	4.6	4.1	4.0
D	4.4	4.5	3.1	3.3	2.7	2.6	3.9	3.8	4.3	4.6	3.8	3.7
E	4.3	4.2	2.6	2.6	2.5	2.2	3.3	3.2	4.6	4.6	3.8	4.4
F	4.5	4.6	2.7	2.9	2.7	2.7	3.6	3.6	4.8	4.4	4.3	3.9
G	4.3	4.3	3.09	2.9	2.5	2.6	3.5	3.9	4.0	3.9	3.5	3.4
H[a]	3.8	3.1	1.7	1.7	1.7	1.8	2.1	2.2	3.6	3.5	3.1	2.9
I[a]	4.0	4.0	2.0	2.0	2.0	2.0	2.0	2.0	4.0	4.0	4.0	4.0
Mean		4.3		2.6		2.4		3.2		4.3		3.8
Reproducibility		0.58		0.48		0.39		0.67		0.49		0.49
CV, %		13.3		18.4		16.2		21.1		11.4		12.8
Repeatability		0.15		0.08		0.17		0.18		0.20		0.28
CV, %		3.5		2.8		6.9		5.8		4.7		7.3
Excluding Outlying Laboratories												
Mean		4.5		2.8		2.5		3.5		4.4		3.8
Reproducibility		0.32		0.25		0.17		0.30		0.37		0.35
CV, %		7.3		8.8		6.7		8.6		8.4		9.1
Repeatability		0.07		0.09		0.10		0.22		0.17		0.22
CV, %		1.4		3.2		4.2		6.3		4.0		5.7

[a] Outlying laboratory.

Table 2. Titratable acidities as % HCl × 10^{-3} in corn syrups, using 0.05N NaOH to pH 7.0 end point

	Sample											
Lab.	1	4	2	12	3	6	5	11	7	9	8	10
A[a]	12.0	12.4	4.4	4.2	4.2	4.4	4.7	4.6	9.6	10.4	8.6	8.6
B	10.3	10.3	2.8	2.9	2.8	2.9	3.9	4.1	8.3	8.5	7.1	7.1
C[a]	12.2	12.3	3.7	4.8	3.7	3.6	4.6	5.1	9.9	10.1	8.5	8.5
D	11.5	11.6	4.2	4.4	4.0	3.3	5.2	4.6	9.3	10.1	8.2	8.1
E	10.4	10.4	3.0	2.8	2.7	2.7	4.2	3.7	9.3	9.1	7.7	7.8
F	10.3	10.7	3.4	3.1	3.0	3.1	3.8	4.0	8.7	8.6	8.2	7.9
G	10.9	11.0	3.5	3.4	3.1	3.1	4.2	4.6	8.6	8.8	7.4	7.4
H[a]	10.5	9.7	2.6	2.3	3.1	2.5	2.9	2.9	8.7	8.4	7.1	7.0
I[a]	11.0	10.0	2.0	2.0	2.0	2.0	3.0	3.0	8.0	8.0	7.0	7.0
Mean		11.0		3.3		3.1		4.1		9.0		7.7
Reproducibility		0.86		0.87		0.69		0.76		0.76		0.63
CV, %		7.8		26.3		22.0		18.6		8.4		8.2
Repeatability		0.33		0.29		0.23		0.25		0.29		0.08
CV, %		3.0		8.8		7.4		6.1		3.2		1.1
Excluding Outlying Laboratories												
Mean		10.7		3.4		3.1		4.2		8.9		7.7
Reproducibility		0.53		0.59		0.40		0.47		0.60		0.45
CV, %		4.9		17.7		12.9		11.1		6.2		5.8
Repeatability		0.13		0.4		0.23		0.29		0.28		0.11
CV, %		1.2		4.1		7.4		6.9		3.1		1.4

[a] Outlying laboratory.

pressed in thousands, so that actual acidities from pH 6.0 to 8.3 were in the range 0.002–0.030% HCl for all samples, and no higher than 0.015% HCl for the high fructose samples. At such low levels, interlaboratory coefficients of variation of 20% are acceptable.

Overall, the data showed substantially greater variabilities at pH 8.3 end point than at either pH 6.0 or 7.0 end point. Visual end point, using phenolphthalein, showed the highest variability of all, in line with the smaller sample and higher normality of alkali.

Table 3. Titratable acidities as % HCl × 10^{-3} in corn syrups, using 0.5N NaOH to pH 8.3 end point

	Sample											
Lab.	1	4	2	12	3	6	5	11	7	9	8	10
A[a]	29.7	23.2	16.1	26.1	16.4	20.3	23.5	25.7	23.4	27.1	21.2	21.2
B[a]	24.7	24.5	6.5	7.1	6.7	6.8	8.7	7.7	17.7	17.6	13.0	13.1
C	31.9	24.5	15.5	13.9	12.8	12.4	13.2	13.4	22.4	22.7	16.2	16.3
D[a]	33.2	33.2	14.4	16.0	13.9	12.8	15.9	13.8	21.0	24.5	18.6	17.7
E	26.6	28.1	8.6	8.8	7.3	8.2	10.7	9.0	21.7	21.0	15.0	14.6
F	26.2	25.8	6.3	6.7	6.2	6.7	7.8	7.0	19.5	19.3	14.6	14.1
G	28.9	29.3	10.7	10.1	9.3	9.6	10.8	11.4	19.5	21.8	14.4	14.7
H	29.2	29.1	11.9	9.2	10.7	10.2	9.9	7.8	19.9	18.7	14.3	14.1
I[a]	26.0	26.0	6.0	7.0	8.0	8.0	7.0	6.0	18.0	18.0	12.0	13.0
Mean		28.1		11.2		10.4		11.6		20.7		15.5
Reproducibility		3.0		5.3		3.9		5.6		2.7		2.7
CV, %		10.8		47.3		38.9		48.6		12.8		17.7
Repeatability		1.59		2.5		1.5		1.0		1.4		0.4
CV, %		5.7		22.3		14.4		8.6		6.7		2.5
Excluding Outlying Laboratories												
Mean		28.6		10.2		9.3		10.1		20.6		14.8
Reproducibility		2.1		3.1		2.4		2.3		1.5		.84
CV, %		7.4		30.5		25.7		23.1		7.3		5.7
Repeatability		0.58		1.02		0.40		0.91		0.97		0.4
CV, %		2.0		10.0		4.2		9.0		4.7		1.6

[a] Outlying laboratories.

Table 4. Titratable acidities as % HCl × 10^{-3} in corn syrups, using 0.1N NaOH to phenolphthalein end point

	Sample											
Lab.	1	4	2	12	3	6	5	11	7	9	8	10
B	25.2	25.6	7.4	6.2	6.6	6.5	7.4	7.5	16.5	16.5	13.6	13.6
D[a]	49.7	31.1	12.3	15.9	11.8	8.9	10.1	12.5	22.0	23.5	20.8	21.7
E	29.2	28.1	8.0	7.3	5.8	4.7	7.3	8.0	14.6	20.4	13.9	17.5
F[a]	20.6	20.2	4.5	4.7	4.1	4.5	5.4	5.3	15.6	15.3	13.1	13.0
H	43.4	39.7	9.1	9.1	9.2	9.4	9.8	9.2	22.9	20.7	19.6	19.9
I	22.0	22.0	4.0	5.0	4.0	5.0	8.0	10.0	16.0	13.0	13.0	14.0
Mean		29.7		7.8		6.7		8.4		18.1		16.1
Reproducibility		9.96		3.66		2.65		2.14		3.70		3.62
CV, %		33.5		46.9		39.5		25.6		20.5		22.4
Repeatability		5.49		1.15		0.95		0.94		2.04		1.11
CV, %		18.5		14.8		14.2		11.2		11.3		6.9
Excluding Outlying Laboratories												
Mean		29.4		7.0		6.4		8.4		17.6		15.6
Reproducibility		8.60		1.97		2.14		1.15		3.53		3.07
CV, %		29.2		28.1		33.5		13.7		20.1		19.6
Repeatability		1.4		0.6		0.5		0.8		2.4		1.3
CV, %		4.7		8.6		8.3		9.3		13.9		8.5

[a] Outlying laboratory.

Different shapes of the titration curves for high fructose and hydrolysate syrup samples were in evidence at pH 7.0, where the individual reproducibilities of HFCS samples 2-12, 3-6, 5-11 were worse than the individual reproducibilities of the hydrolysate samples 1-4, 7-9, 8-10, because the HFCS curves moved from their inflection points at neutrality. This is equivalent to saying that 2 types of samples were analyzed with different effects depending on end point pH. At pH 6.0 end point; however, titratable acidity values and the reproducibility coefficients of variation were similar for all samples.

Repeatabilities followed reproducibilities through this study, ranging from ½ to ⅕ of the values of the latter.

It is recommended that electrometric titration to pH 6.0, using a 100 g sample and 0.005N NaOH, be adopted official first action, to replace the current AOAC method for all corn syrups, **31.217**.

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ALCOHOLIC BEVERAGES

Alcohol Proof Determination from Absolute Specific Gravity (20°C/20°C) Using Oscillating U-Tube Digital Density Meter with Programmable Calculator

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Proof determination using a digital density meter has been difficult at temperatures other than 60°F because no single table for conversion exists as does for apparent specific gravity. The instrument operates more satisfactorily and temperature stability is easier to achieve at temperatures closer to ambient. Because 20°C is widely accepted by the scientific community as the temperature for calibration of equipment and standardization of tests, that temperature was chosen as the basis for the program the author has written to convert specific gravity (20°C/20°C) to proof. AOAC Table 52.003 was the source of data for this program. A table relating specific gravity (20°C/20°C) to proof was also generated from the formula of the International Organization of Legal Metrology (IOLM). Proofs determined using the calculator program and the tables of AOAC, IOLM, and NBS compare favorably.

The oscillating U-tube density meter (Mettler/Paar DMA) is the best available tool for measuring density of liquids. It is more convenient to use and requires a smaller sample size than the next best available tool, the pycnometer. The DMA is more precise and more accurate than the pycnometer (1). The major limiting factor is the cost of the equipment, which can probably be justified when high sample throughput is required, i.e., it is fast compared with the pycnometer. The DMA also allows the possibility of direct readout of proof when coupled with a programmable calculator.

Several papers have been published concerning the use of the DMA for proof determination of distilled spirits (1–5). Although proof of spirits in the United States is defined as twice the percent by volume of ethanol at 60°F (15 5/9°C) (6), the use of a temperature closer to ambient temperature for the DMA is more convenient because the fogging of the DMA window that occurs at some ambient relative humidities is eliminated, and control of temperature is improved. Twenty degrees Celsius was chosen as the temperature basis for this paper for the following reasons: (1) The above criteria are met. (2) This temperature is used by the European Economic Community for alcohol determination, thus enabling simple conversion to European % alcohol at 20°C because the density at that temperature is then known. (3) This temperature is generally accepted by the scientific community for standardization, e.g., volumetric glassware.

Use of the DMA in collaborative studies (4, 5) for proof determination has presented a problem in conversion of the specific gravity (T/T) ($SG^{T/T}$) so determined to proof (T = temperature of measurement). The official practice in proof determination by pycnometer has been to use the AOAC table (7) for this conversion. It was quite natural to use the same table for the conversion to proof of DMA-determined $SG^{T/T}$. The AOAC table was set up, for convenience, to include correction for the buoyant density difference between brass weights and sample so that the user is not required to make this correction for every determination. The DMA, however, yields absolute density or SG, depending on the calibration, i.e., the DMA yields the same result as determination in vacuo. The AOAC table, consequently, cannot be used without first eliminating the buoyant density correction. Furthermore, there exists no table that relates $SG^{20/20}$ to U.S. proof or to % v/v ethanol at 60°F such as the AOAC table does for apparent $SG^{20/20}$. Such tables do exist for $SG^{60F/60F}$ (8, 9). The present work was undertaken to address the above problems and to take full advantage of the automation possible with the DMA 55D digital density meter to obtain U.S. proof as a direct readout from the instrument via a program running in an attached calculator.

Experimental

Apparatus

All computer processing was performed on a Data General Corp. (DGC) Nova 3/4 minicom-

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puter equipped with 32K, 16-bit words of memory and a DGC 10-megabyte hard disk drive. The computer programs were written in DGC ALGOL. The equipment configuration described in this paper for the determination of U.S. proof of ethanol-water solutions is the Mettler/Paar DMA 55D precision digital density meter with and without the Mettler/Paar SP2 automatic sampler and interfaced to an HP 97S programmable calculator via an HP binary coded decimal (BCD) interface. (Use of the calculator is explained by the manufacturer's literature.)

Procedure

U.S. proof and % v/v ethanol of ethanol–water solutions were determined from $SG^{20/20}$ data, using the HP 97S calculator program herein described and tables of the AOAC (7), National Bureau of Standards (NBS) (8), and International Organization of Legal Metrology (IOLM) (10). The IOLM table contained in this paper was generated by the author using the formula on which the IOLM tables are based (10).

Program for DMA 55D/HP 97S/SP2

The DMA 55D digital density meter can be equipped with the HP 97S programmable calculator which accepts data from the DMA over a BCD interface. Data transfer is initiated when the "PRINT" button on the DMA is pressed. The automatic sampler, SP2, is also available from Mettler/Paar for attachment to the DMA, in which case the data transfer is initiated automatically by the SP2. The program for proof determination developed by the author for the HP 97S will accommodate either configuration.

To write the program for the HP 97S, it was necessary to reduce the AOAC table for apparent $SG^{20/20}$ ($SG^{20/20}_{app}$) vs % v/v ethanol (60°F) to an equation that would fit the storage capacity of the calculator. A decision was made to limit the absolute error between the AOAC table and the equation fit to the table to ±0.01% v/v ethanol. An attempt was made to fit these data to one polynomial equation, using the method of least squares. This proved impossible because the HP 97S has only 10-digit precision, and the limited number of data registers did not allow for storage of the coefficients of a polynomial of sufficiently high order to fit the irregular $SG^{20/20}_{app}$ vs % v/v ethanol (60°F) curve for ethanol–water solutions. The problem was solved by fitting the data to a series of overlapping equations, none of which was higher than fourth order. The coefficients were rounded to 10-digit precision and stored on 3 magnetic data cards.

Because the SG obtained from the DMA cannot be used to enter the AOAC table directly, the program was written to incorporate the buoyant density correction in reverse, i.e., the $SG^{20/20}$ was converted to $SG^{20/20}_{app}$ by rearranging the equation for the buoyant density correction. The equation used for the buoyant density correction at constant temperature (8) is as follows:

$$M = W[1 + [\rho(d_2 - d_1)]/[d_2(d_1 - \rho)]]$$

where M = weight in vacuo; W = apparent weight in air; ρ = density of air; d_1 = density of body; d_2 = density of weights. To apply this equation to the conversion of $SG^{20/20}$ to $SG^{20/20}_{app}$, M can be replaced by d_1 and W by d_{app}, i.e., apparent density. Since $SG^{20/20} = d_1/d_{water}$ and $SG^{20/20}_{app} = d_{app}/d_{app(water)}$ then

$$SG^{20/20}_{app} = SG^{20/20}\left|\frac{d_{water}\,(d_2)}{d_{app(water)}\,(d_2 - \rho)}\right| - \left|\frac{d_2\rho}{d_{app(water)}\,(d_2 - \rho)}\right|$$

$$\text{or } SG^{20/20}_{app} = SG^{20/20}\,a - b$$

Assuming that brass weights are used (d_2 = 8.40 g/mL) and air is at 50% relative humidity (ρ = 0.001199429 g/mL, see below for calculation), then a = 1.001207702 and b = 0.001203002.

The value, 0.997172771 g/mL, for $d_{app(water)}$ was obtained by dividing the weight (in g) in air of 1 gal. water at 20°C, viz., 3774.630 g, by the volume (in mL) it occupies, which is identical in value to the weight (in g) in vacuo of 1 gal. water at 4°C, viz., 3785.332 g. The value, 0.998234501 g/mL, for d_{water} was calculated in a similar fashion from the same table, using the weight of 1 gal. water in vacuo at 20°C, viz., 3778.649 g (8).

The density of air at 20°C and 50% relative humidity was calculated using the following formula (11):

$$\text{Density of moist air} = [0.0012929 \times (P - 0.3783e)\,273.13]/760(273.13 + t)$$

where P = atmospheric pressure in mm Hg; t = air temperature in °C; e = pressure contribution of water vapor in air. In this case, P = 760 mm Hg; t = 20°C; e = 0.5(17.535 mm Hg).

A normal printout of the program is shown in (1) of Figure 1. The program is designed to detect the presence of the SP2 in the system. The input to the calculator is negative when the SP2 is present and positive when it is absent. This

```
          Tape Printout      Explanation
          -------------      -----------

(1)             1.   ***  << Sample #
              0.95165  ***  << SG(20/20) (in vacuo)
              0.95160  ***  << SG(20/20) (in air)
                38.72  ***  << % EtOH (v/v) @ 60°F
                77.44  ***  << Proof

(2)             2.   ***  << Sample #
              0.00123  ***  << SG(20/20) air
                               No further output for this sample.
                               Sample # will be repeated for next sample.

(3)             3.   ***
              0.99001  ***
              0.99000  ***
            111111111. ***  << This output indicates that this sample
                               is not in the range of the currently loaded
                               data card.  The string of 1's indicates that
                               data card #1 must be loaded for proof deter-
                               mination of this sample.  No further output
                               for this sample.  Sample # will be incremented
                               for next sample.

(4)             4.   ***
              1.00010  ***
              1.00010  ***
            888888889. ***  << Indicates that this sample cannot use this
                               program to calculate proof, i.e., it falls
                               outside the program range (0.7905<SG<1.0000).
                               Sample # will be incremented for next sample.
```

Figure 1. Sample of possible printout from HP 97S program.

distinction is essential to proper functioning of the error-handling routine. An error occurs when the $SG^{20/20}$ is beyond the range of the current data card or not within the range of the program, i.e., $0.7905 < SG^{20/20}_{app} < 1.0000$. In the absence of the SP2, the program will cause the word "Error" to be displayed when the $SG^{20/20}$ is out of program range or will flash the number of the data card that should be inserted when the $SG^{20/20}$ is out of the range of the current data card. Because these 2 situations would cause loss of data with the SP2, the program simply prints a number string to indicate the error and continues with the next sample. Items (3) and (4) of Figure 1 are examples of error handling when the SP2 is in the system. An additional advantage of the SP2 is that air is determined between samples, providing a check on the reproducibility of the determination.

Table for $SG^{20/20}$ vs Proof from IOLM Formula

To develop a table relating proof to $SG^{20/20}$, the easiest course was to use the formula of the IOLM, which contains % w/w and temperature (°C) as independent variables and density (kg/cu. m) as the dependent variable. We wanted to make $SG^{20/20}$ the index of the table as $SG^{20/20}_{app}$ is for the AOAC table. Instead of trying to solve the IOLM formula for % w/w, Newton's approximation was incorporated into an iterative procedure in an ALGOL program to arrive at the solution for each $SG^{20/20}$ in the table. After an initial guess for the desired value, Newton's approximation employs the first derivative of a function to arrive at the next best guess (12). Rearranged for this purpose, Newton's formula is as follows:

$$x_1 = x_0 - f(x_0)/f'(x_0),$$

where x_1 is the next best guess and x_0 is the initial guess. Using 2 initial guesses, x_0 and x_1,

$$(f(x_1) - f(x_0))/(x_1 - x_0)$$

can be used as an approximation of $f'(x_0)$, giving

$$x_2 = x_0\, f(x_0)(x_1 - x_0)/(f(x_1) - f(x_0))$$

Since Newton's approximation is given for the solution of the roots of $f(x) = 0$, and for our purposes $f(p) = SG^{20/20}$ (which is not zero), where p = % w/w, the substitutions of $f(p_0) - SG^{20/20}$ for $f(x_0)$ and $f(p_1) - SG^{20/20}$ for $f(x_1)$ were necessary. Otherwise, all x's were replaced by p's. The result is

$$p_2 = p_0 - (f(p_0) - SG^{20/20}) \times (p_1 - p_0)/(f(p_1) - f(p_0))$$

Each successive iteration replaced p_1 with the last p_2 and p_0 with the last p_1 and was allowed to proceed that way until:

$$|f(p_2) - SG^{20/20}| \leq 0.0000005$$

Convergence to the correct solution occurred within 5 iterations when the initial approximations were 0% w/w and 100% w/w.

Use of NBS Tables to Arrive at Proof from $SG^{20/20}$

To verify the accuracy of the buoyant density correction and the IOLM table described above, proof was also determined using the NBS tables (8). This required entry into 2 tables. After conversion of $SG^{20/20}$ to density (20°C), Table 2 was entered to obtain % w/w and then Table 5 was entered to convert % w/w to % v/v at 60°F, which was doubled to obtain proof. Linear interpolations were used with both tables.

Results

Table 1 is a comparison of proofs (at approximately 10° proof intervals) obtained from $SG^{20/20}$ and $SG_{app}^{20/20}$, using the HP 97S program, AOAC table, NBS tables, and IOLM table described above. Column 2 data were calculated by the HP 97S program from column 1, using the buoyant density correction described above. Column 3 results were obtained by entering the AOAC table with the data from column 2. Column 4 data were obtained from the HP 97S program. The data in columns 5 and 6 were obtained from the indicated tables by using data from column 1. Columns 7 and 9 display the erroneous results from using $SG^{20/20}$ data from column 1 to enter the AOAC table and the HP 97S program (without the buoyant density correction), respectively. The respective deviations from true proof are indicated in columns 8 and 10, i.e., column 8 = column 7 − column 3 and column 10 = column 9 − column 4.

Discussion

Inspection of the data in Table 1 reveals that, with few exceptions, the values in columns 3, 4, and 5 are within 0.01° proof. In no case do they differ by more than 0.03° proof. Those data that differ by 0.03° proof may do so for the following reasons: an interpolation error when using the NBS tables caused by 2 linear interpolations in-

Table 1. Comparison of proof tables of AOAC, NBS, and IOLM with calculator program using specific gravity (in vacuo and apparent) at 20°C

SG 20/20	SG_{app} 20/20	Proof				Proof from SG AOAC	Error	Proof from SG program	Error
		AOAC	Program	NBS	IOLM				
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
0.99900	0.99900	1.32	1.33	1.33	1.32	1.32	0.00	1.33	0.00
0.99300	0.99300	9.68	9.67	9.69	9.73	9.68	0.00	9.66	−0.01
0.98700	0.98699	19.10	19.07	19.10	19.07	19.08	−0.02	19.06	−0.01
0.98100	0.98098	29.36	29.35	29.35	29.42	29.32	−0.04	29.32	−0.03
0.97500	0.97497	40.46	40.44	40.47	40.45	40.40	−0.06	40.39	−0.05
0.97000	0.96997	49.55	49.55	49.56	49.58	49.50	−0.05	49.50	−0.05
0.96400	0.96396	59.76	59.75	59.76	59.76	59.70	−0.06	59.69	−0.06
0.95700	0.95695	70.31	70.31	70.31	70.29	70.24	−0.07	70.25	−0.06
0.95000	0.94994	79.53	79.53	79.53	79.56	79.46	−0.07	79.46	−0.07
0.94100	0.94093	90.13	90.12	90.13	90.13	90.06	−0.07	90.05	−0.07
0.93200	0.93192	99.68	99.68	99.69	99.69	99.60	−0.08	99.61	−0.07
0.92200	0.92191	109.53	109.52	109.53	109.52	109.44	−0.09	109.44	−0.08
0.91100	0.91090	119.68	119.67	119.68	119.67	119.58	−0.10	119.58	−0.09
0.89900	0.89888	130.12	130.11	130.12	130.13	130.02	−0.10	130.02	−0.09
0.88700	0.88687	140.02	140.02	140.03	140.03	139.92	−0.10	139.92	−0.10
0.87400	0.87385	150.20	150.21	150.20	150.20	150.08	−0.12	150.10	−0.11
0.86100	0.86084	159.80	159.79	159.80	159.80	159.68	−0.12	159.68	−0.11
0.84600	0.84582	170.21	170.21	170.21	170.20	170.08	−0.13	170.09	−0.12
0.83100	0.83080	179.78	179.78	179.80	179.77	179.66	−0.12	179.66	−0.12
0.81300	0.81278	189.91	189.93	189.92	189.90	189.80	−0.11	189.81	−0.12
0.79100	0.79075	199.90	199.88	199.90	199.87	199.80	−0.10	199.79	−0.09

stead of polynomial interpolations; minor rounding errors from the incorporation of the buoyant density correction into columns 3 and 4 (compare differences in columns 3 and 4 with the differences in columns 7 and 9 for $SG^{20/20}$ 0.98700 and 0.97500).

One of the requirements imposed on the fitting of equations developed for the HP 97S program was that the program duplicate the AOAC table within 0.01% v/v (0.02° proof). A comparison of columns 7 and 9 shows that this criterion was met.

It can be seen that column 6 (IOLM table) deviates from the others by as much as 0.07° proof (at $SG^{20/20}$ = 0.98100). This discrepancy may well be due to the different data sources for the IOLM formula (10). The IOLM formula is based on 7 different international sources, including NBS data (13). It seems that an arbitrary decision was made to include all official international tables extant at the time the formula was developed. All other tabulated proof results are based on the data of Osborne et al. of NBS (13), a decision which is fully vindicated by a IUPAC paper on the subject (14). An obvious discrepancy between the IOLM tables and the NBS table is the density of 100% ethanol at 20°C. The IOLM table reports it as 0.78926 g/mL (converted from kg/cu. m) vs the NBS table value of 0.78934 g/mL. This discrepancy accounts for a difference of 0.03° proof at the high end of the table. Some of the preceding discussion may seem to be much ado about nothing, but definitive reference tables should be exactly comparable.

The use of the AOAC table for the direct conversion of $SG^{20/20}$ to % v/v ethanol (60°F) and proof in refs 2–5 accounts for much of the discrepancy between the DMA and pycnometer results in those studies. The remaining differences may well be due to problems with operation of the DMA (notably temperature stability) and the inherently greater accuracy and precision of the DMA over the pycnometer. (For a discussion of these problems, see Kovar (1).)

In one of those studies, the use of air–sample pairs is suggested for DMA standardization (2). This is not recommended because it would involve standardizing the DMA by a method (the pycnometer) which is less accurate than the DMA (1). The use of $SG^{20/20}_{air}$ = 0.00120, as long as the ambient pressure is 760 mm Hg ± 30 mm Hg, has little effect on the calibration of the DMA provided that the B constant is calculated using T_{air}, the period of oscillation for air determined by the DMA (1). The other standard in the pair should be water or a similar substance for which the density is well known and which is capable of reproducible purity.

Conclusion

The HP 97S program for the conversion of $SG^{20/20}$ data to proof for ethanol/water solutions generated from the Mettler/Paar DMA 55D density meter compares favorably with other methods of proof determination. There is no operator intervention (such as table lookup) necessary because all required data (sample number, $SG^{20/20}$, $SG^{20/20}_{app}$, % v/v ethanol (60°F) and proof) are printed on the calculator paper tape. The calculator program developed simulates a table for conversion of $SG^{20/20}$ to proof which did not heretofore exist.

A copy of the program listing with documentation and associated data is available from the author.

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Determination of Authenticity of Sake by Carbon Isotope Ratio Analysis

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Sake samples are fractionally distilled so that the resultant ethanol concentration of the distillate is approximately 95%. Determination of ^{14}C by liquid scintillation counting on the ethanol fraction differentiates alcohol produced by fermentation from synthetic ethanol produced from fossil fuel sources. $^{13}C/^{12}C$ stable isotope ratio analysis (SIRA) is capable of detecting alcohol from a cane or corn source in sake samples. This analysis also shows the addition of corn or cane sugar before or after fermentation of the sake.

The measurement of the radioactive carbon isotope ^{14}C provides a direct means of differentiating products derived from fossil fuels and those derived from present-day natural products. The source of ^{14}C present in organic plant material is the uptake of atmospheric $^{14}CO_2$, a process that has been relatively constant for many thousands of years.

Because synthetic ethanol is produced from dead carbon (petroleum products void of ^{14}C), radiocarbon liquid scintillation counting is an ideal method for differentiating natural and synthetic compounds (1, 2). The biosynthesized compound has a relatively constant level of ^{14}C because of the uniform distribution and uptake of this carbon isotope by plants in the form of $^{14}CO_2$. The amount of ^{14}C present in an ethanol sample, compared with the value established for a 100% natural ethanol standard, enables determination of the amount of natural and synthetic (petroleum-derived) ethanol.

Plants differ by fixing CO_2 by one of 3 photosynthetic metabolic pathways: Calvin synthesis, Hatch-Slack pathway, or Crassulacean acid metabolism. Each biochemical mechanism results in natural products with different $^{13}C/^{12}C$ ratios. Therefore, the $^{13}C/^{12}C$ ratio can determine the CO_2 fixation pathway of the carbon in a given natural product. In the Calvin synthesis, which is common to most plants, e.g., rice, CO_2 is fixed by the carboxylation of ribulose-1,5-diphosphate (RUDP) (3) to give 3-phosphoglycerate. The carbon isotope fractionation occurs at this enzymatic step (4). The Hatch-Slack pathway is used by some plants to fix atmospheric CO_2 by the carboxylation of phosphoenolpyruvate (PEP) (5), an enzymatic reaction that occurs with low carbon isotope fractionation (6). Plants that fix atmospheric CO_2 by this pathway are *Zea mays* (corn), *Saccharum*, and *Cymbopogon citratus* (lemon grass) (7). The Crassulacean acid metabolic pathway is characteristic of *Vanilla plainfolia*. Carbon assimilation occurs at night by carboxylation of PEP, with accumulation of malic acid which is decarboxylated in the presence of light; liberated CO_2 is fixed by RUDP. Carboxylation of PEP at night varies under the influence of environmental factors, and can be estimated by carbon isotope composition (8).

In U.S. federal regulations, sake is a unique alcoholic beverage product. Pure brewed sake is defined as rice wine and yet is taxed as beer. The addition of distilled spirits to brewed sake is prohibited. Furthermore, the addition of sugar to rice before fermentation is not allowed in the manufacture of brewed sake (9).

Carbon-14 analysis of sake will clearly demonstrate whether synthetic ethanol has been added to the sake; SIRA analysis of the ethanol and sugar components of sake will determine which of the following statements is true: (*1*) The sake is a naturally fermented product obtained solely from rice. (2) Spirits from cane and/or corn have been added. (3) Cane and/or corn sugar were added to the sake before fermentation. (4) Corn and/or cane sugar were added following fermentation.

Experimental

Ethanol Purification or Isolation

A 750 mL sample is distilled using a fractionating column with approximately 25–50 theoretical plates. The column is washed with water and dried under vacuum before use. The column head temperature is brought to 78°C. The split ratio in the reflux head is adjusted to 5:1 (5

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parts back to the column and 1 part collected). Approximately 150 mL distillate is collected. Ethanol concentration in the distillate is checked by a densitometer and should be approximately 95% v/v ethanol.

Sugar Purification or Isolation

Liquid-liquid extraction.—Approximately 100 mL of sample remaining in the distilling flask after ethanol purification is taken for sugar analysis. The sample is subjected to liquid–liquid extraction with 25 mL chloroform. The aqueous fraction is washed with 2 additional 25 mL portions of chloroform, and all chloroform fractions are discarded. The residue is now ready for column chromatography.

Column Preparation.—Twenty to twenty-five g anion exchanger (analytical grade, AG-1-X2, 100–200 mesh, Bio-Rad Laboratories, Richmond, CA) is placed in a 2 cm id × 30 cm column. A plug of silanized glass wool is placed on top of the anion exchanger. The column is charged with 200 mL 6N HCl, and then washed with water until the effluent is brought to pH 6. The sample is placed on the column and the effluent is collected in a beaker. Water is evaporated from the effluent and the remaining portion (sugar) is collected for $^{13}C/^{12}C$ analysis.

Stable Isotope Ratio Analysis (SIRA).—Ethanol is oxidized in a closed oxygen atmosphere maintained at 600°C. Resulting carbon dioxide and water are collected in a liquid nitrogen trap and oxygen is removed by vacuum. The nitrogen trap is heated in dry ice and carbon dioxide is recondensed in a sample tube. The $^{13}C/^{13}C$ isotope ratio of carbon dioxide from the sample tube is measured with a mass spectrometer equipped with a dual inlet system and double collector.

Isotope deviations in δPDB ^{13}C units are expressed as parts per thousand (per mil, ‰) compared with reference standard Pee Dee Belemnite (PDB) calculated according to following equation:

$$^{13}C(‰) = \left| \frac{(^{13}C/^{12}C \text{ sample})}{(^{13}C/^{12}C \text{ standard})} 1 \right| \times 1000$$

SIRA tests were performed at Geochron Laboratories Division, Krueger Enterprises, Inc., 24 Blackstone St, Cambridge, MA.

^{14}C Isotope Analysis

^{14}C is determined by using a low level liquid scintillation counting technique. The isolated ethanol is ignited, and the combustion products, CO_2 and water vapor, are separated. CO_2 is converted to acetylene via lithium carbide. Acetylene gas is then catalytically trimerized to benzene. ^{14}C is determined on the benzene sample dissolved in liquid scintillation cocktail (10). A special Teflon lead-shielded vial is used to reduce background of the counter (11). The discrimination windows to the liquid scintillation counter (Picker 220) are set between the E_{max} energy of ^{3}H and ^{14}C to eliminate the introduction of any ^{3}H activity into the ^{14}C determination. Counting efficiency for the ^{14}C energy window setting was 68% with background av-

Table 1. ^{14}C and $\delta^{13}C$ values for ethanol in domestic and imported sake

Sample	Sake	^{14}C		$\delta^{13}C$, ‰
		Measured,[a] dpm/g carbon	Corrected,[b] dpm/g carbon	
1	domestic	ND[c]	ND	−27.5
2	imported	15.76	18.30	−26.6
3	imported	15.74	18.30	−26.8
4	imported	15.54	18.10	−25.4
5	domestic	15.65	18.20	−25.0
6	imported	14.76	17.20	−26.8
7	domestic	15.87	18.70	−25.0
8	imported	15.74	18.30	−24.9
9	imported	15.84	18.60	−26.8
10	imported	ND	ND	−25.3
11	imported	15.23	17.72	−25.7
Mean		15.57	18.16	
SD		0.36	0.46	
CV, %		2.32	2.53	
3σ (SD)		1.08	1.38	

[a] Based on New England Nuclear Standard (toluene).
[b] Calculated from NBS 95% Oxalic Acid Standard.
[c] ND = not determined.

Table 2. $\delta^{13}C$ values (‰) for sugar and ethanol in imported and domestic naturally fermented sake

Sample	Sake	Ethanol	Sugar
13	imported	−26.9	−25.4
14	imported	−27.0	−25.9
15	imported	−27.3	−25.8
16	imported	−24.7	−24.4
17	imported	−26.1	−25.1
18	imported	−26.1	−25.2
19	domestic	−26.5	−24.6
20	imported	−26.3	−25.1
21	imported	−26.3	−24.9
22	imported	−26.6	−25.3
Mean		−26.2	−25.2
	SD	0.84[a]	0.47
	CV, %	3.19[a]	1.87
	3σ SD	2.51[a]	1.41

[a] Includes values for Tables 1 and 2.

eraging 3.12 counts/min. A liquid scintillation standard is prepared from ^{14}C-labeled toluene (New England Nuclear).

Carbon-14 analyses were performed by the Center for the Applied Isotopes Studies, University of Georgia, 110 Riverbend Rd, Athens, GA.

Results and Discussion

Samples of sake were first analyzed for ^{14}C content to determine whether the ethanol was a product of natural fermentation. Previous work (12) has shown that mean dpm/g carbon value for synthetic ethanol is 0.167. The mean dpm/g carbon value for ethanol in a naturally fermented sake, based on the New England Nuclear Standard, was 15.57 with a standard deviation of 0.36 and a coefficient of variation of 2.32%, and when corrected to a 95% Oxalic Acid Standard (NBS), a mean value of 18.16 (SD 0.46 and CV 2.53%) (Table 1). All samples fell within the 3 σSD limits shown in previous work (12) for naturally fermented ethanol.

$\delta^{13}C$ deviations for the sugar/ethanol fractions that were derived from samples of naturally fermented sake are shown in Table 2. These results gave mean values of −26.2 and −25.2‰ $\delta^{13}C$ for the ethanol and sugar fractions, respectively. The standard deviations for sugar and ethanol were 0.84 and 0.47, respectively. These values also indicated that the sugar fraction of naturally fermented sake was approximately one unit more positive than was the ethanol fraction.

Table 3. $\delta^{13}C$ values (‰) for various mixtures of 95% v/v ethanol derived from sake and corn

% ethanol from sake	% ethanol from corn	Experimental	Theoretical
100	0	−27.5	—
75	25	−22.7	−23.6
50	50	−20.0	−19.8
25	75	−14.9	−15.9
0	100	−12.0	—

Table 4. $\delta^{13}C$ values (‰) for ethanol and sugar in imported sake, showing sugar is derived from rice but ethanol is partially derived from cane or corn-base distillate

Sample	Ethanol	Sugar
23	−22.0	−25.0
24	−21.4	−24.5
25	−23.5	−25.4
26	−21.1	−25.4
27	−23.1	−24.9
28	−21.5	−25.0
29	−22.7	−25.0
30	−22.1	−25.1
31	−22.2	−25.0
32	−23.0	−25.2

The next step was to establish the ability to distinguish ethanol fermented from rice and ethanol fermented from corn and cane sugar. Mixtures of 95% ethanol derived from sake/or corn distillate were prepared as follows: 75/25, 50/50, and 27/75. SIRA analyses of the mixtures gave the following $\delta^{13}C$ values: −22.7, −20.0, and −14.9‰, respectively. These results (Table 3) compare favorably with the calculated $\delta^{13}C$ values.

Table 4 shows $\delta^{13}C$ values obtained for the ethanol and sugar fractions for 10 samples of imported sake. The $\delta^{13}C$ values of the sugar fraction of these samples indicated that no corn or cane sugar was added to these sakes either before or after fermentation of the rice. The $\delta^{13}C$ value of each ethanol fraction clearly indicated that ethanol from cane or corn distillates was added to the sake. The majority of these samples yielded values greater than −22.7‰, indicating that at least 25% of the ethanol present was derived from cane or corn distillate.

$\delta^{13}C$ values for ethanol and sugar fractions in 4 additional samples, one domestic and 3 imported, are shown in Table 5. $\delta^{13}C$ values for the ethanol fraction of the domestic sake indicate that the ethanol was derived solely from rice. $\delta^{13}C$ values for the sugar fraction clearly indicated the addition of cane and/or corn sugar to the sake after fermentation. The $\delta^{13}C$ values for the ethanol fraction for the 3 imported samples showed clearly the presence of ethanol from cane and/or corn. The $\delta^{13}C$ values for the sugar

Table 5. $\delta^{13}C$ values (‰) for ethanol and sugar in imported and domestic sake, showing time of addition of sugar derived from corn or cane (before or after fermentation)

Sample	Sake	Ethanol	Sugar	Sugar added
33	imported	−22.1	−21.3	before
34	imported	−21.2	−20.6	before
35	imported	−20.1	−18.6	before
36	domestic	−27.5	−17.5	after

fraction, like those obtained from the sugar fractions of the naturally fermented sake (Table 2), yielded values for $\delta^{13}C$ of approximately one unit more positive than the corresponding alcohol fraction. Therefore, we conclude that corn and/or cane sugar was added to the rice before fermentation.

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METALS AND OTHER ELEMENTS

Rapid Direct Determination of Lead in Evaporated Milk by Anodic Stripping Voltammetry Without Sample Pretreatment

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The method presented describes the direct determination of lead in evaporated milk in which the milk ashing step prior to analysis is eliminated. Digital instrument readout units are μg Pb/mL milk. Total analysis time after instrument calibration is less than 3 min per sample. Range of the method is 0.05-1.0 ppm lead in milk, and precision of the method expressed by relative standard deviation of duplicate pairs ranged from 30% at 0.1 μg/mL to 3% at 1.0 μg/mL of lead in milk. The method compares favorably with the AOAC official first action anodic stripping voltammetric method (25.074). In addition, the method appears to work equally well for skim evaporated milk, sweetened condensed milk, and nonfat powdered dry milk when the latter two are reconstituted with water according to product label instructions. Recovery and interference studies are presented.

Increasing government regulation of the trace metal contaminant lead in milk and the consequent need for a higher testing frequency by manufacturers have highlighted the need for a faster and easier method for its determination in evaporated milk. AOAC methods **25.063** and **25.074** (1), while precise and accurate, both suffer from several limitations. Both methods require a 28 h dry ashing step before analysis can proceed. In practice, the dry ashing step can extend over 3 working days. Turnaround time is slow. Both methods require extensive sample handling and the concomitant need for highly skilled technicians. Chances for sample contamination due to long exposure to the atmosphere and from reagents are high.

The method described here overcomes those disadvantages and offers another distinct advantage. As written, it requires the use of a lead-free reagent called Metexchange®. This reagent is offered by the manufacturer in prepackaged cuvets. Intralaboratory comparisons need not suffer from the normal variability in reagent quality. In addition, it has already been shown that evaporated milk standards may be prepared and used for at least 40 days without loss of sample integrity with respect to lead (2). Thus, a central facility may directly control and standardize tests run at several secondary facilities by preparing its own standards for use in calibration instead of using the more general procedure described in the method.

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Experimental

Principle

Lead is determined by anodic stripping voltammetry performed directly on a mixture of milk and Metexchange reagent. The reagent decomplexes the lead and makes it available for anodic stripping voltammetric (ASV) analysis. Elimination of organic material in the sample by customary wet or dry ashing procedures is not necessary. An aliquot of a well shaken sample is added to reagent in a prepackaged cuvet. The cuvet is placed on the electrode cell head, and 96 s later, the lead concentration in μg Pb/mL milk can be read from the digital display. The concept of an automated ASV analyzer and the use of Metexchange reagent have been described elsewhere (3-6). The method is given in detail in the following paper (7).

Reagents

All chemicals used were reagent grade. Standard solutions of metal ions other than those specifically described were purchased as atomic absorption stock standard solutions, 1-10 mg/mL in dilute acid. All solutions used in interference studies were tested for lead content and, if necessary, were made lead-free by electrolysis over a mercury pool at −1.2 V vs Ag/AgCl reference. Copper and tin standards were prepared from 99.999% pure metals obtained from ROC/RIC, Sun Valley, CA. All solutions used in interference tests were buffered to pH 1.5, using KCl-HCl buffer.

Unless noted otherwise, all potentials (voltages) specified in the method and subsequently in this paper, are determined vs the saturated

sodium chloride, silver, silver chloride reference electrode.

All glassware and Nalgene polyethylene bottles used in this study were rendered lead-free by rinsing well in deionized water, soaking in 1% $HClO_4$ for a minimum of 24 h, and rinsing well in deionized water once again. No apparatus was used unless the 1% $HClO_4$ soak solution contained less than 5 ppb Pb.

Sample Preparation

It was necessary to spike evaporated milk samples with lead to determine and study the range of this method. When this was done, 500 mL portions of evaporated milk were stabilized against deproteinization by the addition of 0.5 mL 36.1% formaldehyde. The evaporated milk was then spiked with the appropriate amount of lead from the stock solution described above. The spiked milk was stirred 2 h at room temperature and then drawn off into 6 oz Nalgene polyethylene bottles. Samples prepared in this manner were kept refrigerated at 2–5°C when not in use or being shipped.

Other spiked samples were prepared from unsterilized evaporated milk that had never previously been canned. Their method of preparation has been described elsewhere (8).

Procedures

Every analysis parameter was varied to determine the optimum setting. This was done by analyzing multiple aliquots of 4 representative types of milk samples as each parameter was varied. The 4 types of milk samples were regular evaporated milk, skim (less than 0.25% butterfat) evaporated milk, nonfat dry milk reconstituted according to the package instructions, and sweetened condensed milk diluted 1:1.5 with deionized water.

Once the analysis conditions were determined, samples were prepared at both Carnation Co. Research Laboratories, Van Nuys, CA, and Environmental Sciences Associates, Inc., Bedford, MA. Samples were traded and analyzed by 3 methods. Method A: direct method described above; Method B: anodic stripping voltammetry, AOAC **25.074** (1); Method C: anodic stripping voltammetry as follows: Wet ash 0.5 g aliquots of well shaken sample to dryness with 2.0 mL acid mixture (H_2SO_4–HNO_3–$HClO_4$ (1 + 24 + 24 by volume)). Reconstitute each sample with 5 mL 0.06 F $HClO_4$ and heat to 90°C for 15 min. Cool to room temperature and conduct ASV analysis by plating at −0.75 V at the composite mercury graphite electrode for 15–20 min. Strip to +0.100 V at 60 mV/s linear ramp rate.

Method B was used in the initial studies by Carnation Research Laboratories and Methods A and C were used by Environmental Sciences Associates, Inc., laboratories.

Results and Discussion

Initial or plating potential studies showed no increase in lead signal above −1.040 V. A value of −1.090 V was chosen to allow for operator error in setting this parameter, and possible reference electrode drift.

Final potential: The correct value for the final potential was dependent on the copper content of the sample. Too negative a final potential effected buildup of copper in the thin film mercury electrode and a consequent negative error in the lead result. A value of −0.110 V was chosen to prevent small errors in this setting from creating the situation just described.

Sweep rate: The slowest sweep rate and the narrowest integration window that would still encompass the lead peak arising from a sample containing 1.5 μg Pb/mL were determined by running spiked samples and slowly varying this parameter. An optimum value of 10.5 mV/step was determined. A 0.040 V error in correct integration set point can still be made without seriously affecting lead results.

Recorder set point: The correct recorder set point is one at which the dedicated computer within the instrument can pick a baseline current against which proper integration of the lead signal can be made. This point must not occur while another metal is stripping from the electrode. It was determined that even in the presence of 5.0 μg Cd/mL, correct lead values could be obtained if the recorder set point was −0.725 V. This level of cadmium in evaporated milk is at least 500 times higher than normally encountered.

Integration set point: The optimum value for the integration set point was determined to be −0.490 V using the method outlined in the manufacturer's instructions (6). We found that the optimum value can vary ±0.020 V from week to week and that the overall accuracy of the method was more dependent on this parameter than on any other. Consequently, we optimized this particular parameter every week. The procedure described by the manufacturer is not tedious and is equivalent to adjusting the flame position when tuning an atomic absorption spectrophotometer.

Analysis time: Duplicate determinations of spiked evaporated milk samples ranging in lead

Table 1. Maximum allowable levels of possible interferences

Interferent	μg/mL in evaporated milk	Type
Cadmium	5.0	negative
Calcium	>30 000	none detected
Chromium	>20 000	none detected
Copper	3.0	negative
Indium	30	negative
Thallium	0.5	negative
Tin	150	positive
Acetate ion	>100 000	none detected
Chloride ion	>100 000	none detected
Phosphate	≃10 000	negative

concentration from 0.02 to 1.4 μg Pb/mL were performed at both 1 and 2 min plating times. No differences in results were obtained other than a slight increase in precision below the 0.1 μg Pb/mL level. The slight increase in precision did not warrant doubling the analysis time. For 1 min plating times, relative standard deviation for duplicate pairs ranged from 30% at 0.1 μg Pb/mL to 3% at 1.0 μg Pb/mL.

Reaction time between evaporated milk and Metexchange reagents: Duplicate aliquots of spiked samples ranging from 0.02 to 1.4 μg/mL were mixed with reagent and allowed to stand for 24 h. These samples were then analyzed together with aliquots of the same spiked milks mixed with the reagent less than 1 min before analysis was initiated. No differences were obtained and all values obtained agreed well with those for the same samples by Methods B and C.

Interference studies: These studies were performed by adding aliquots of the different types of milk described earlier to the reagent. Lead analyses were performed as the interference under study was added in ever increasing concentration. Analog output was used as a visual confirmation of the digital signal. Results are shown in Table 1.

With the exceptions of tin, calcium, and phosphate, the concentration levels of the interferences studied are not normally found in evaporated milk. These levels might well be encountered in contaminated sample handling equipment and/or in reagents and standards improperly prepared. Average concentrations of calcium and phosphate in evaporated milk are 2300 and 2000 μg/mL, respectively. This method of analysis will be relatively insensitive to large changes in concentrations of both calcium and phosphate.

The tin content of canned evaporated milk ranges from 40 to 60 μg/mL. Both in vitro and in vivo studies confirmed that accurate lead analysis could be performed in the presence of as much as 150 μg tin/mL milk. At tin concentrations greater than 150 μg/mL, apparent lead response as indicated by the digital readout begins to increase dramatically.

In essence then, this method appears to be relatively free of interferences at the normal levels one would expect to encounter in milk samples.

Results of Comparison with Other Methods

Two sets of samples were run by the method under study and by 2 comparison methods. All determinations were made in duplicate and the averages are reported in Table 2. The first set consisted of Samples 1–30. Samples 1–27 were real samples of canned evaporated milk. Samples 28–30 were spiked evaporated milk controls whose preparation has been described earlier (8). Over 200 of these controls had previously been analyzed by Method B and the average value found was 0.125 ± 0.03 μg/mL.

The remaining 10 samples identified by a combination letter/number code in Table 2 were prepared by the Associate Referee. The spiking procedure was described earlier. Sample M1 was powdered nonfat dry milk reconstituted as per label instructions. Sample M2 was a portion of Sample M1 spiked to a level of 0.55 μg Pb/mL milk over the base sample lead content. Sample N1 was canned evaporated milk and Sample N2 was a portion of N1 spiked to a level of +0.22 μg Pb/mL over the base sample lead content. Sample P1 was canned sweetened condensed milk diluted by adding 3 parts condensed milk to 2 parts deionized water. The sample was then spiked to a level of +0.62 μg Pb/mL over the base sample lead content. Unspiked sample was not retained. Sample Q2 was canned evaporated milk measured at 0.07 μg Pb/mL by the direct Method A. This milk was spiked at +0.16 μg Pb/mL over an approximate base level of 0.07 μg Pb/mL to give a total of approximately 0.23 μg Pb/mL milk. Sample Q3 was the same milk spiked to a target level of 0.46 μg Pb/mL milk. Samples R1 and R2 were canned evaporated milk spiked to target levels of 0.95 and 1.26 μg Pb/mL, respectively. Sample S1 was canned evaporated skim milk (less than 0.25% butterfat) spiked to a target level of 0.39 μg Pb/mL.

With the exception of Samples 28–30, the target levels inserted in Table 2 are approximations with potential errors of ±0.05 μg lead/mL. They are inserted in Table 2 as guideposts only.

Table 2. Comparison of lead values obtained by 3 methods on 40 evaporated milk samples (μg Pb/mL milk)

Sample	Method A	Method B	Method C	Sample	Method A	Method B	Method C	Target lead level
1	0.11	0.16	0.14	21	0.15	0.13	0.11	
2	0.11	0.18	0.16	22	0.10	0.15	0.10	
3	0.15	0.18	0.15	23	0.09	0.15	0.08	
4	0.08	0.12	0.08	24	0.10	0.17	0.12	
5	0.10	0.12	0.11	25	0.01	0.08	0.02	
6	0.11	0.15	0.13	26	0.10	0.15	0.07	
7	0.11	0.13	0.07	27	0.12	0.14	0.13	
8	0.11	0.15	0.14	28[a]	0.17	0.12	0.11	0.125
9	0.13	0.14	0.13	29[a]	0.17	0.12	0.10	0.125
10	0.06	0.05	0.03	30[a]	0.14	0.10	0.08	0.124
11	0.07	0.10	0.06	M1	0.02	0.01	0.01	—
12	0.11	0.15	0.12	M2[a]	0.61[b]	0.45[b]	0.70[b]	0.57
13	0.05	0.07	0.04	N1	0.11	0.11	0.14	—
14	0.07	0.11	0.04	N2[a]	0.35	0.33	0.37	0.33
15	0.03	0.07	0.04	P1[a]	0.74	0.72	0.72	0.70
16	0.06	0.07	0.05	Q2[a]	0.25	0.19	0.23	0.23
17	0.07	0.08	0.05	Q3[a]	0.50	0.41	0.53	0.46
18	0.09	0.10	0.07	R1[a]	1.09[b]	0.93[b]	1.11[b]	0.95
19	0.11	0.08	0.11	R2[a]	1.41[b]	1.07[b]	1.43[b]	1.26
20	0.08	0.07	0.04	S1[a]	0.45	0.40	0.41	0.39

[a] Control or spiked sample, see text.
[b] Not used in statistical analysis, see text.

Wider than normal variation among the 3 methods and the approximate target levels were obtained for Samples M2, R1, and R2. These results were not used in the overall statistical analysis of the data. Table 3 contains the results of linear regression analysis comparing various combinations of the methods. When methods are compared on the last 10 values, Samples M2, R1, and R2 were included in the comparison.

A *t*-test for paired values was also performed among the 3 methods for Samples 1–20, M1, and N1 and for the 37 pairs not footnoted in Table 2. Over the range 0.01–0.74 μg lead/mL (37 pairs), there was no significant difference between Method A and Method B or between Method A and Method C at the 95% confidence level. There was a significant difference between Method B and Method C at that confidence level. Over the range 0.01–0.18 μg Pb/mL (Samples 1–30, M1, and N1), there was no significant difference between Method A and Method C, but the differences were significant between Methods A and C or Method B.

Since Methods B and C both require that the

Table 3. Results of regression analysis on 37 pairs

Parameter	Method A (Y) vs Method B (X)	Method A (Y) vs Method C (X)
Intercept, μg/mL	−0.023	+0.011
Slope	1.15	1.03
Correlation coefficient	0.964	0.982
Standard error of estimate, μg/mL	0.038	0.027
Average for all samples by Method A, μg/mL	0.145	0.145
Average for all samples by Method B, μg/mL	0.147	—
Average for all samples by Method C, μg/mL	—	0.130

	SD of dupl. pairs, μg/mL	Variance (μg/mL)[a]
Method A	0.018	0.0003
Method B	0.021	0.0004
Method C	0.028	0.0008

[a] Variance for each of the 3 methods is lower than that of 0.0010 reported for Method B earlier.

Table 4. Comparison of lead values obtained by Method A and Method B on evaporated milk samples (μg Pb/mL milk) [a]

Sample	Method A 3010—Direct	Method B AOAC **25.074**
1	0.125	0.120
2	0.125	0.130
3	0.115	0.115
4	0.160	0.125
5	0.105	0.140
6	0.110	0.135
7	0.135	0.120
8	0.085	0.105
9	0.125	0.145
10	0.165	0.155
11	0.135	0.150
12	0.125	0.140
13	0.115	0.150
14	0.235	0.255
15	0.125	0.150
16	0.170	0.180
17	0.170	0.155
Av.	0.1367	0.1452

[a] Both methods performed in one laboratory. Duplicate analyses were performed for each sample by each method and averages are reported in this table.

sample be ashed to destroy organic matter, it seemed unlikely that the differences between methods was due to incomplete exchange of the lead in the Metexchange reagent used in Method A. Methods A and C had been used by one laboratory (ESA, Inc.) and Method B was used by another laboratory (Carnation Research). The results in Tables 2 and 3 and the *t*-test data suggested that what had been obtained was a comparison between laboratories and not comparison of methods. Indeed, the differences in Table 2 between Methods A and C and Method B are more typical of those obtained during routine interlaboratory comparisons (2).

A separate set of samples was run at Carnation Research using Methods A and B. These results are tabulated in Table 4. No significant difference between Method A and B was found at the 95% confidence level. Hence, it would appear that differences between laboratories was the cause of the earlier disagreement between Method B and Methods A and C.

The direct method appears to show a positive bias above 1.0 μg Pb/mL. However, if so, Method C suffers essentially the same bias—an unlikely situation. Samples spiked with large amounts of lead were also spiked with large amounts of nitric acid used to preserve the lead standards. It is possible that too much acid caused deproteinization and resulted in sample inhomogeneity. In any event, method bias at or above 1.0 μg Pb/mL levels, if real, does not seriously detract from Method A because extremely few, if any, real samples will ever be found to contain concentrations of lead in excess of 1.0 ppm.

Practical Limit of Measurability

For 1 min plating times, the instrumentation was found to reliably measure 5 ng lead. This translates to 0.025 μg Pb/mL for a 200 μL aliquot size. Good agreement among Methods A, B, and C was obtained for Sample M1 at 0.01 μg/mL; however, this was the only comparison sample with lead concentration below 0.05 μg/mL, so it is safer to assume a practical limit of measurability of 0.05 μg Pb/mL milk.

Conclusions

The direct method represents a viable alternative to presently accepted methods. It is as precise and accurate as **25.074** (1, 8) and much faster and easier to perform.

Recommendations

It is recommended that a collaborative study of the direct method be undertaken with a view toward adoption as official first action, if successful.

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Direct Determination of Lead in Evaporated Milk and Apple Juice by Anodic Stripping Voltammetry: Collaborative Study

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A method for the direct determination of lead in evaporated milk and in fruit juice with no prior sample digestion was successfully collaborated by 13 laboratories. The anodic stripping voltammetric (ASV) method studied consisted of adding 0.2 mL aliquots of evaporated milk or 0.3 mL aliquots of fruit juice to 2.9 mL of a dechelating reagent, Metexchange. The reagent-sample mixture is then analyzed for lead by ASV with no further sample preparation. Each collaborator received 24 samples, 2 each at 5 different levels (0.07–0.70 ppm for spiked evaporated milk and 0.09–0.87 ppm for spiked apple juice) along with duplicate practice samples of labeled lead content at each of 2 levels for each sample type. All unknowns were coded with random numbers. Approximately 69% of the reporting laboratories had never analyzed either evaporated milk or fruit juice for lead. Average time between receipt of samples and reporting of results was 1.6 days for all laboratories. The pooled variations between duplicate determinations for apple juice and evaporated milk were 0.00059 and 0.00043, respectively. The method was adopted official first action for both fruit juice and evaporated milk.

This report describes the collaborative study of methods for the direct determination of lead in evaporated milk and in fruit juices by anodic stripping voltammetry (ASV). Developmental work concerning the methods has been described in detail (1, 2). Briefly, an analysis for lead in evaporated milk or juice is accomplished by adding 0.2 mL milk or 0.3 mL juice to a dechelating reagent which immediately releases lead from its complexes with the organic molecules of the sample. (Metexchange® reagent is a patented proprietary formulation manufactured for sale by Environmental Sciences Associates, Inc., 45 Wiggins Ave, Bedford, MA 01730. U.S. patent No. 4,374,041.) The sample–reagent mixture is then analyzed for lead by ASV with no further preparation necessary.

There are at present 2 methods for the analysis of lead in evaporated milk (3, 4). These were both adopted as official final action AOAC methods at the 89th annual meeting in October 1975. Both methods are accurate, although somewhat time consuming. In each method, a 25 g sample is dry ashed. Ashing according to instructions (3) requires a minimum of 30 h to complete and also requires an oven for drying and a muffle furnace for ashing. At present, no collaboratively studied and adopted methods exist for the analysis of lead in fruit juices.

By comparison, the methods described herein accomplish the analysis in less than 3 min with equivalent accuracy and precision.

Collaborative study of these methods had been recommended when they were first described. However, because of alternative methods, it was not felt necessary to complete the process required for adoption by AOAC. Renewed interest in this area of food chemistry (5–8) caused reassessment of our earlier position.

Collaborative Study

Juice Samples

Apple juice packed in clear bottles was obtained from the local supermarket. All bottles of juice bore the same pack and date codes, and bottles with sediment were excluded. Seven 500 mL portions of the juice were spiked with 0.5 mL 36% formaldehyde to retard spoilage. Each 500 mL portion was then spiked with varying amounts of 1000 ppm lead standard solution. The lead solution was prepared only moments

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The recommendation of the Associate Referee was approved by the General Referee and Committee E and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1979) 62, 380; 415.
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before the spiking operation in 0.2F lead-free sodium citrate solution at pH 5.5. This was done to prevent acid-catalyzed decomposition of the apple juice. Spiked apple juice samples were stirred magnetically for 4 h at room temperature and then bottled and refrigerated; 48–64 h after refrigeration, each bottle was warmed to room temperature and then 7 mL portions of juice were placed in lead-free polyethylene cuvets (9) and capped. Approximately 70 cuvets containing each fruit juice sample were prepared. Each cuvet of each test sample was labeled with a different 4 digit number obtained using a random number program on a desk-type computer calculator. Practice samples were labeled with their known lead concentrations.

Milk Samples

Raw nonsterile evaporated milk was fortified with lead (in the form of $Pb(NO_3)_2$). One mL 36% formaldehyde/L milk was added to retard spoilage. Samples were blended with a Kraft nonaerating mixer for 1 min after addition of lead and formaldehyde. Seven 0.5 L portions of milk at 7 different lead concentrations were prepared by David Sakai of Carnation Co. Research Laboratories, Van Nuys, CA. These 7 samples were shipped to the Associate Referee in Burlington, MA. About 7 mL portions were pipetted into decontaminated polyethylene cuvets and numbered in the same manner as were the apple juice samples.

After the tubing operation, duplicate 0.5 mL aliquots were withdrawn from 5 tubes of each juice and milk sample and tested by the Associate Referee for lead. The results agreed with the target spiking levels within experimental error and homogeneity was also assured.

Two samples each of 5 lead levels for both juice and evaporated milk were then mailed to each collaborator. Two practice samples each of juice and evaporated milk with stated lead content were included. The collaborators were also sent approximately 100 tubes each containing 2.9 mL Metexchange reagent, a proprietary formulation that the collaborators could not be expected to prepare. The Associate Referee retained 2 of the complete sets of samples sent to collaborators. These were stored at room temperature, 20–27°C, for the duration of the collaborative study. Other sets of samples were kept refrigerated, 2–6°C, for the same period of time. Samples shipped to the collaborators were not insulated or refrigerated. Average delivery time was 3.3 days and the mean temperature of the samples was estimated at 27°C.

Instructions to Collaborators

Collaborators were requested to analyze each sample and report their results within 10 days. They were supplied with separately written methods for each sample type. The collaborators were also supplied with report forms for each set of samples. Collaborators were not advised that duplicate samples were sent, but they were asked to analyze each sample only once. They were also asked not to analyze the unknown samples until they had achieved correct results for the labeled practice samples.

Lead in Milk and Fruit Juices

Anodic Stripping Voltammetry

First Action

25.080 ***Apparatus***

(**a**) *Voltammetric analyzer.*—With staircase anodic stripping ramp and graphite electrode coated with thin film of Hg. Capable of measuring 5 ng Pb in presence of dissolved O. (Solns cannot be deaerated.) Peak area integration desirable. ESA Model 3010A Trace Metals Analyzer (Environmental Sciences Associates, Inc., 45 Wiggins Ave, Bedford MA 01730), or equiv.

(**b**) *Micropipets.*—50, 100, 200, and 300 μL, pos. displacement type. (SMI or Drummond, available from supply houses, or equiv.).

25.081 ***Reagents***

(Use deionized H_2O to prep. std solns. Prep. and store solns in same Pyrex vol. flasks. Do not wash flasks with strong acids between use; just rinse 3 times with deionized H_2O. Always prep. same soln in same flask.)

(**a**) *Lead releasing reagent.*—Contg <1 ppb Pb. Acid soln of cation able to displace Pb from sample. Metexchange reagent (Environmental Sciences Associates, Inc.), or equiv.

(**b**) *Lead std solns.*—(*1*) *Stock soln.*—1 mg/mL. Prep. as in **25.065(d)**.(1). (*2*) *Intermediate soln.*—10 μg/mL. Pipet 1 mL stock soln into 100 mL vol. flask contg 1.0 mL HNO_3 and ca 50 mL H_2O. Mix, and dil. to vol. Prep. each week. After soln is prepd 6 times in same flask, it is stable 1 month. (*3*) *Working soln for fruit juice detn.*—3 μg/mL. Pipet 30 mL soln (*2*) into 100 mL vol. flask contg 0.7 mL HNO_3 and ca 50 mL H_2O. Mix, and dil. to vol. (*4*) *Working soln for evaporated milk detn.*—1 μg/mL. Prep. as in (*3*), using 10 mL soln (*2*) and 1.0 mL HNO_3. Working solns are stable 3 days. After being prepd 5

times in same flask, they are stable 2 weeks. (5) *Calibration solns.*—Evapd milk or fruit juice of type being detd, and contg ≥0.5 ppm added Pb.

25.082 *Determination*

Calibrate instrument according to manufacturer's directions. Mix aliquot of sample with releasing reagent, (**a**), and perform detn according to manufacturer's instructions. Data for ESA analyzer are as follows:

	Juice	Milk
Initial potential, V	−1.025 ± 0.005	−1.090 ± 0.005
Final potential, V	−0.100 ± 0.005	−0.100 ± 0.005
Sweep rate, mV/step	14.0 ± 0.05	10.50 ± 0.05
Integration set points, V	−0.490 ± 0.005	−0.490 ± 0.005
Sample size, μL	300	200

Run control or spiked sample with each 15–20 analyses in a series.

Results and Discussion

Samples were sent to 20 collaborators; 13 reported results. Six laboratories that did not report results had nontechnical reasons, i.e., vacation schedules, unexpectedly heavy workloads, general shutdowns, etc. The seventh nonreporting collaborator could not achieve the correct results on the practice samples. It was determined that this collaborator's samples had decomposed during transit. Coincidentally, this collaborator was geographically the farthest away from the Associate Referee's laboratory (approximately 1500 miles). Reporting collaborators averaged 1.6 days between receipt of samples and reporting of results.

Reported results for juice and milk samples are given in Tables 1 and 2, respectively. All results for one collaborator, F, were excluded from the statistical analysis of variance because this collaborator did not follow the method as outlined. Three other pairs of values footnoted in Tables 1 and 2 were also excluded from the analysis of variance according to the rejection test of Dixon and Massey at the 95% confidence level (10). Results of tests on samples sent to collaborators by laboratories of the Associate Referee and Carnation Co. are shown in Table 3.

Table 1. Collaborative results for direct ASV determination of lead in apple juice

	Target lead level, ppm				
Lab.	0.09	0.17	0.33	0.55	0.87
A	0.08	0.19	0.36	0.48	0.91
	0.07	0.21	0.34	0.47	0.90
B	0.15	0.22	0.33	0.54	0.85
	0.10	0.19	0.32	0.54	0.88
C	0.11	0.19	0.42	0.60	1.00
	0.07	0.13	0.39	0.55	0.96
D	0.07	0.17	0.34	0.54	0.87
	0.09	0.14	0.30	0.55	0.88
E	0.10	0.23	0.39	0.65	1.03
	0.12	0.22	0.40	0.64	1.03
F[a]	0.14	0.21	0.36	0.62	0.96
	0.08	0.24	0.39	0.73	0.99
G	0.09	0.14	0.34	0.56	0.94
	0.08	0.18	0.35	0.57	0.92
H	0.10	0.17	0.38	0.58	1.03
	0.05	0.15	0.37	0.59	0.93
I	0.11	0.15	0.33	0.55	0.86
	0.09	0.23	0.36	0.55	0.93
J	0.09	0.16	0.35	0.64	0.98
	0.10	0.15	0.35	0.62	0.96
K	0.11	0.19	0.38	0.58	0.87
	0.11	0.20	0.38	0.53	0.92
L	0.07	0.15	0.24	0.48	0.71[b]
	0.06	0.12	0.30	0.38	0.89
M	0.11	0.21	0.25	0.52	0.96
	0.08	0.13	0.31	0.49	0.91

[a] All values for this collaborator excluded from statistical analysis, see text.
[b] Superscript pairs excluded from statistical analysis, see text.

Table 2. Collaborative results for direct ASV determination of lead in evaporated milk

Lab.	Target lead level, ppm				
	0.07	0.16	0.30	0.50	0.70
A	0.11	0.18	0.28	0.45	0.55
	0.10	0.19	0.29	0.46	0.56
B	0.11	0.24	0.35	0.59	0.75
	0.11	0.21	0.26	0.58	0.79
C	0.11	0.19	0.32	0.47	0.58
	0.11	0.16	0.27	0.44	0.59
D	0.15	0.18	0.34	0.59	0.75
	0.12	0.19	0.32	0.56	0.78
E	0.10	0.17	0.31	0.54	0.66
	0.09	0.19	0.35	0.55	0.66
F[a]	0.18	0.63	0.26	0.45	0.62
	0.10	0.18	0.30	0.46	0.45
G	0.12	0.19	0.36	0.49	0.63
	0.09	0.18	0.31	0.47	0.63
H	0.11	0.16	0.30	0.45	0.61
	0.09	0.14	0.29	0.45	0.57
I	0.11	0.19	0.32	0.53	0.70
	0.13	0.19	0.34	0.55	0.63
J	0.14	0.20	0.29	0.47	0.58
	0.13	0.20	0.45[b]	0.45	0.60
K	0.11	0.19	0.29	0.48	0.75
	0.13	0.19	0.31	0.50	0.79
L	0.10	0.18	0.31	0.69[b]	0.70
	0.11	0.22	0.34	0.52	0.70
M	0.08	0.20	0.34	0.54	0.64
	0.09	0.18	0.31	0.50	0.61

[a] All values for this collaborator excluded from statistical analysis, see text.
[b] Superscript pairs excluded from statistical analysis, see text.

Results tabulated in Table 3 show that the samples prepared for collaborative study did not decompose or otherwise deteriorate with respect to lead content over the life of the study. These data also suggest that the collaborators were better able to analyze the samples than was the Associate Referee able to prepare them at the intended target levels.

Results of the statistical analysis of collaborator values are shown in Tables 4 and 5 for apple juice and evaporated milk, respectively. For 3 levels in the evaporated milk study and for one level in

Table 3. Results of reference laboratories and methods

Method	Target lead level, ppm				
	0.09	0.17	0.33	0.55	0.87
Apple Juice Samples					
Direct method 1 week before collaboration	0.090	0.170	0.345	0.575	0.925
Direct method 2 weeks after collaboration	0.088	0.173	0.325	0.555	0.913
Method D (11, 12)	0.086	0.165	0.331	0.559	0.861
	0.07	0.16	0.30	0.50	0.70
Evaporated Milk Samples					
Direct method 1 week before collaboration	0.085	0.150	0.275	0.490	0.630
Direct method 1 week after collaboration	0.095	0.150	0.290	0.493	0.630
Method D (11, 12)	0.096	0.163	0.295	0.505	0.630
AOAC 25.074	0.103	0.160	0.280	0.460	0.640

Table 4. Statistical analysis for lead in apple juice collaborative study

Statistic	Target lead level, ppm				
	0.09	0.17	0.33	0.55	0.87
Overall average, $\bar{x}$	0.092	0.176	0.345	0.550	0.933
Number of labs	12	12	12	12	11
Number of determinations	24	24	24	24	22
SD of overall average, $SD_{\bar{x}}$	0.0152	0.0069	0.0088	0.0129	0.0120
95% Confidence region for average	0.062 0.122	0.162 0.190	0.327 0.363	0.524 0.576	0.909 0.957
Var. between labs, SD_L^2	0.00518	—	0.00154	0.00326	0.00215
Var. within labs, SD_E^2 (repeatability or error)	0.000379	0.00115	0.00040	0.00070	0.00106
Reproducibility ($SD_L^2 + SD_E^2$)	0.0056	0.0012	0.0019	0.0040	0.0032
Repeatability, CV, % ($SD_E/\bar{x}$) 100	21.2	19.3	5.8	4.8	3.5
Pooled error variance = 0.00059, pooled error precision = 0.024 ppm					

the apple juice study, the between-laboratories variance component, SD_L^2, was not significant at the 95% confidence level. For the other target levels, the between-laboratories variance component was significant at the 95% confidence level. Differences in performance ability are normally found (4), and these reported here are not exceptional.

The within-laboratory variance component, SD_E^2, is excellent for both types of samples at all levels. For comparison, reference 4 describes the collaborative studies and results obtained in support of adoption of AAS and ASV methods **25.063** and **25.074,** respectively.

As expected, coefficient of variance is highest at the lowest level and then decreases at higher levels for both sample types.

A remarkable feature of this study is the 1.6 day average turnaround time achieved by the collaborators, in spite of the fact that approximately 69% of the collaborators had never before analyzed food samples for lead. This accomplishment, together with the overall success of the study in terms of accuracy and precision, suggests exceptional ruggedness and simplicity of the methods.

With the exception already noted, no collaborator reported any difficulties with the methods. Collaborator B used 250 μL aliquots of both sample types instead of 200 μL milk and 300 μL juice as requested. Collaborator B's results did not differ significantly from the other collaborators, so actual choice of aliquot size within limits does not seem to be a factor. All analysis parameters were optimized before the collaborative study and we see no reason to change the method with respect to aliquot size.

One collaborator commented on the relatively small sample aliquot size taken for analysis. He suggested that contamination by pipetting technique or reagent quality can be crucial factors when microanalytical work is done. Examination of the data suggests that pipetting technique is not a significant factor except, perhaps, at the lowest levels studied. Reagent quality is a relatively unimportant factor, also. There are only 2 reagents used in the method, i.e., Metexchange reagent and a lead standard solution. The manufacturer of the Metexchange reagent guarantees overall quality of the reagent at less than 1 ppb lead, whether supplied in individual 2.9 mL aliquots or in 500 mL bottles. Prepackaged 2.9 mL volumes of reagent can, of course, vary slightly in lead content from tube to tube, but the manufacturer reports less than a 0.1% event error rate for significant contamination in any one tube.

The Associate Referee recommends that all analyses using this method be run in duplicate and that an average result be reported. Should duplicate values differ significantly, a third aliquot can then be analyzed to confirm the lower or higher result.

Conclusion and Recommendation

A successful collaborative study of the direct method for the analysis of lead in evaporated milk and fruit juice has been accomplished. The methods studied are as precise and accurate at relevant lead concentrations as the accepted AOAC-ASV method for lead in evaporated milk. Simplicity and ruggedness have been demonstrated by the rapid turnaround time and accuracy achieved by analysts unfamiliar with the methods described above.

It is recommended that the direct ASV method for the analysis of lead in fruit juice and evapo-

Table 5. Statistical analysis for lead in evaporated milk collaborative study

Statistic	Target lead level, ppm				
	0.07	0.16	0.30	0.50	0.70
Overall average, $\bar{x}$	0.110	0.188	0.314	0.505	0.659
Number of labs	12	12	11	11	12
Number of determinations	24	24	22	22	24
SD of overall average, $SD_{\bar{x}}$	0.0035	0.0041	0.0058	0.0109	0.0161
95% confidence region for average	0.103 0.117	0.180 0.196	0.302 0.326	0.483 0.527	0.627 0.691
Var. between labs, SD_L^2	—	—	—	0.0024	0.0057
Var. within labs, SD_E^2	0.00029	0.00040	0.00074	0.00024	0.00050
Reproducibility	0.00029	0.00040	0.00074	0.0026	0.0062
Repeatability, CV, %	15.5	10.6	8.7	3.1	3.4
Pooled error variance = 0.00043, pooled error precision = 0.021					

Statistic	Target lead level, ppm				
	0.06	0.16	0.22	0.27	0.95
Overall average, $\bar{x}$	0.091	0.169	0.214	0.295	0.949
Number of labs	6	6	6	6	6
SD of overall average, $SD_{\bar{x}}$	0.0081	0.0061	0.0094	0.0217	0.0298
Var. within labs, SD_E	0.00064	0.00031	0.00016	0.00295	0.00396
Repeatability, CV, %	27.9	10.4	5.9	18.4	6.6
Pooled error variance = 0.0016, pooled error precision = 0.04 ppm					

rated milk, using Metexchange reagent, be adopted official first action.

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Mixed Acid Solubilization Procedure for Determination of Total Mercury in Food Samples

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A mixed acid solubilization procedure has been developed for the determination of total mercury in food samples. This procedure has eliminated the problem of mercury loss from samples that are high in lipid or carbohydrate content. The solubilization is rapid and quantitative and can be used for a wide range of food items.

A number of digestion procedures can be used in the analysis of food items for mercury (1–11). The most widely used procedure is an acid solubilization (nitric acid and sulfuric acid) of the mercury in the sample followed by cold vapor atomic absorption spectrophotometric (AAS) determination of the total mercury (2, 3). This procedure has been used in our laboratory and has been found to work well for most food samples. However, mercury losses have been observed from food items that are high in lipid or carbohydrate content because these samples char in the presence of sulfuric acid and the mercury is reduced to its elemental state and lost. Although attempts have been made to minimize or eliminate the mercury loss (8–11), they have usually involved the addition of larger quantities of nitric acid, the reduction of the size of the samples used, or the introduction of reagents that could contribute additional residues to the samples. For our work in the Total Diet Program, these options are not acceptable.

The Total Diet Program, conducted by the Food and Drug Administration (FDA) with analyses performed at the FDA District Laboratory in Kansas City, is a surveillance program for monitoring the levels of pesticides, herbicides, industrial chemicals, and individual elements in foods after they have been prepared for consumption. A complete description of the program is presented elsewhere (12). The samples cover the entire range of foods, including some that are very high in lipid or carbohydrate content. For these samples, a minimum sample size of 5 g is needed to maintain a low level of detection. In addition, the volume of reagents used in the preparation of samples for analysis must be kept to a minimum. Because the sample size cannot be reduced and the use of additional reagents would complicate our analyses, we developed a digestion procedure to prevent mercury loss from samples containing high concentrations of lipid or carbohydrate.

Reference to commercial equipment or materials is made to completely describe the work performed. Such references do not in any way constitute an endorsement or recommendation by the Food and Drug Administration.

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Experimental

Apparatus

Block digestion system.—Tecator DS-20 programmable heated block digestion system (Tecator, Inc., Herndon, VA). The system has a working temperature range of 50–450°C. The temperature can be automatically adjusted via the programmable controller. The heating rate is approximately 4°C/min. The block is capable of heating 20 digestion vessels (30 × 4 cm od), each with a working volume of 250 mL. A full evaluation of this system is presented elsewhere (13).

Solubilization Procedure

Samples with low lipid or carbohydrate content.—Place 5 g sample in digestion vessel. Add ca 10 mg vanadium pentoxide to each vessel as catalyst for digestion. Rinse vessel walls with deionized water and add 20 mL HNO_3–H_2SO_4 (1 + 1) to each vessel. Proceed to sample digestion.

Samples with high lipid or carbohydrate content.—Place 5 g sample in digestion vessel. Attach digestion vessels to water condensers. Add 10 mL H_2SO_4 in 1 mL increments through each condenser. (The sample does char, but if the acid is added slowly so that the temperature of the solution remains low and care is taken to prevent the formation of a large solid mass of carbon, the mercury remains in solution.) Add 10 mL 50% hydrogen peroxide solution in 1 mL increments. Let reaction subside before adding next increment. Add 10 mL HNO_3 in 1 mL increments. Rinse condensers with deionized water and remove them. Proceed to sample digestion.

Sample digestion.—Place vessels in block digestion system and heat to 100°C. Maintain temperature at 100°C for 6 min; then increase temperature to 200°C at rate of 4°C/min. Remove vessels from digestion block and let cool.

Table 1. Recovery of mercury [a] from individual food items by nitric acid/sulfuric acid digestion procedure (2)

Item	Sample wt, [b] g	Total Hg found, [b] μg	Rec., %
Sugar	5.07 (5.42)	— (—)	— [c]
Peas	5.34 (5.01)	0.474 (0.004)	94
Fried chicken	5.23 (5.09)	0.496 (0.022)	95
Orange juice	5.15 (6.05)	0.514 (0.016)	100
Peanut butter	4.97 (5.46)	— (—)	— [c]
Corn	5.23 (5.49)	0.512 (0.016)	99
Shrimp	5.07 (5.14)	0.640 (0.152)	98
Beer	5.67 (6.56)	0.526 (0.020)	101
Sausage	5.55 (5.06)	0.468 (0.010)	92
Spinach	6.52 (6.56)	0.526 (0.022)	101
Canned vegetables	5.25 (4.94)	0.520 (0.010)	102
Scalloped potatoes	5.24 (6.60)	0.508 (0.008)	100
Granola	5.42 (5.08)	— (—)	— [c]
Whole wheat bread	5.01 (5.18)	0.235 (0.005)	46 [c]
Meat loaf	5.47 (5.04)	0.490 (0.010)	96
Cantaloupe	5.21 (5.51)	0.564 (0.034)	106
Cheese	5.13 (5.40)	0.490 (0.004)	97
Boiled eggs	5.77 (6.27)	0.490 (0.022)	94
Chicken and vegetables	5.13 (5.19)	0.506 (0.022)	97

[a] 0.500 μg Hg added.
[b] Values for control samples in parentheses.
[c] Sample charred; mercury loss.

Transfer solutions to 100 mL volumetric flasks and dilute to volume with deionized water.

Determination

Analyze samples by cold vapor AAS procedure (2, 3).

Results and Discussion

The official AOAC method of analysis for mercury (2) is quite cumbersome when a large number of samples must be digested simultaneously. The digestion system must be operated manually, and the total time for the digestion of 20 food samples varies from 6 to >8 h, depending on the types of samples analyzed. An evaluation of a heated block digestion apparatus showed that the same sample could be digested via an automated approach in approximately 4 h (14). The difference in time is associated primarily with the operator intervention needed for the AOAC procedure.

Table 1 shows the results obtained from the analysis of a variety of food items by the nitric acid/sulfuric acid solubilization procedure (2). A complete digestion is not necessary to dissolve the mercury in these samples (2, 3). Recoveries are good for all items except peanut butter, sugar, whole wheat bread, and granola. For these foods, recoveries are very low, indicating loss of mercury. Analysis of the same items with the procedure described here for foods with high lipid or carbohydrate content showed complete recovery of the mercury (Table 2). In addition, tests with NBS Standard Reference Materials showed that the procedure gave quantitative recoveries of mercury over the analytical range 0.03–0.95 ppm (Table 3). This is the mercury range normally encountered in the food samples that we analyze.

The nitric acid/sulfuric acid solubilization procedure has not been changed for foods with low lipid or carbohydrate content because this procedure gave good results for these samples (Table 1). For foods with high lipid or carbohydrate content, we found that by keeping the temperature of the sample plus the sulfuric acid solution low and by agitating the solution to prevent the formation of large masses of carbon, the mercury could be kept in solution. Oxidation of the samples with 50% hydrogen peroxide

Table 2. Recovery of mercury [a] from individual food items by sulfuric acid–hydrogen peroxide–nitric acid solubilization procedure [b]

Item	Sample wt, [c] g	Total Hg found, [c] μg	Rec., %
Sugar	5.06 (4.98)	0.446 (0.010)	87
Granola	5.25 (5.12)	0.472 (0.010)	92
Whole wheat bread	5.31 (5.22)	0.488 (0.010)	96
Peanut butter	5.31 (5.34)	0.476 (0.010)	93

[a] 0.500 μg Hg added.
[b] This work.
[c] Values for control samples in parentheses.

Table 3. Recovery of mercury from NBS Standard Reference Materials by sulfuric acid–hydrogen peroxide–nitric acid solubilization procedure[a]

SRM[b]	Sample wt, g	Hg expected from sample, μg	Hg spike, μg	Total Hg found, μg	Rec., %
Albacore Tuna	0.78	0.74	0.00	0.67	91
(0.95 ± 0.10)	0.47	0.45	0.50	0.92	97
	0.63	0.60	0.00	0.58	97
	0.60	0.57	0.50	1.03	96
Spinach	0.93	0.028	0.000	0.026	93
(0.030 ± 0.005)	0.89	0.027	0.500	0.512	97
	1.03	0.031	0.000	0.032	103
	1.03	0.031	0.500	0.524	99
Orchard Leaves	1.04	0.161	0.500	0.610	92
(0.155 ± 0.015)	1.00	0.155	0.000	0.130	84
	1.08	0.167	0.500	0.602	90

[a] This work.
[b] Standard Reference Material; μg Hg/g sample in parentheses.

converts the carbon to carbon dioxide and oxidizes the mercury to the more soluble mercuric ion. In addition, the hydrogen peroxide does not contribute any impurities that would complicate the analyses. Similar procedures have been used previously but the sample size was greatly reduced (10, 11). The nitric acid was added to remove excess hydrogen peroxide, to ensure complete solubilization, and to provide the same solvent composition for food samples with low and high lipid or carbohydrate content.

The solubilization scheme presented here can be used for all the foods in the Total Diet Program. It has proven to be a rapid and quantitative procedure for our analyses for total mercury in food samples.

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COLOR ADDITIVES

Ion-Pair Liquid Chromatographic Determination of Uncombined Intermediates in Three Synthetic Food Colors

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Uncombined intermediates are determined in samples of food colors by reverse phase liquid chromatography with a mobile phase containing tetra-n-butylammonium phosphate. The intermediates are separated from the primary dyes and are quantitated by using known standards. The intermediates naphthionic acid and R-salt (2-naphthol-3,6-disulfonic acid, sodium salt) were determined in spiked samples of Amaranth with recoveries of 98–102%. Similar results were obtained for sulfanilic acid and Schaeffer's salt (2-naphthol-6-sulfonic acid, sodium salt) in Sunset Yellow FCF (FD&C Yellow No. 6) and for sulfanilic acid and PyT (1-(4-sulfophenyl)-3-carboxy-5-hydroxypyrazolone) in Tartrazine (FD&C Yellow No. 5) with recoveries of 96–105%. Spiking levels, in all cases, were in the range 0.05–1.0% total intermediates. The method compares favorably with results obtained by either ion-exchange liquid chromatography or cellulose column chromatography for a number of commercial samples.

Uncombined intermediates are starting materials which may be present in food colors as a result of incomplete reaction during commercial color synthesis. These compounds are considered impurities, and the Canadian government has set limits on their concentration in synthetic colors approved for food use. The permitted levels of total intermediates in Amaranth, Sunset Yellow FCF, or Tartrazine cannot exceed 0.5% by weight. In the past, the methodology has involved a cellulose column/ammonium sulfate procedure (1–3) or thin layer chromatography (4). These methods were considered unsuitable for the rapid separation and quantitation of individual intermediates which may be present in food colors. Liquid chromatography has been shown to be very useful for the determination of food color intermediates by ion exchange (5–9), while ion-pair chromatography has found much use for the separation and quantitation of primary food dyes (10–16) and some subsidiary dyes (17).

We evaluated an ion-pairing technique for the separation of primary food dyes (10) and the determination of subsidiary dyes in individual colors (17). The procedure was suitable in terms of simplicity, speed of analysis, and reproducibility. We have extended this approach to include food color intermediates.

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Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—Waters Associates (Milford, MA) Model 6000A pump, Model 450 variable wavelength detector, and Rheodyne 7125 syringe-loading injection port with 20 μL loop. Operating conditions: flow rate, 1.0 mL/min; detector sensitivity, 0.01 to 0.2 absorbance unit full scale (AUFS); UV light source; ambient temperature; detector wavelength 237 nm for intermediates of Amaranth and Sunset Yellow FCF or 249 nm for intermediates of Tartrazine.

(b) *Liquid chromatography column.*—E. Merck (Darmstadt, GFR) Hibar II LiChrosorb RP-18, 10 μm (4.6 mm × 25 cm) at ambient temperature.

(c) *Recorder.*—Varian (Palo Alto, CA) Aerograph Model 20. Parameters: single pen; 1 mV input; chart speed 0.25 in./min.

(d) *Filter system.*—Millipore (Bedford, MA) 0.45 μm vacuum filter apparatus.

(e) *Tetra-n-butylammonium hydroxide (TBAH) solution.*—AR grade TBAH (BDH Chemicals Ltd, Poole, UK); 40% solution in water (1.54M). Store at ambient temperature in dark.

(f) *Ion-pair reagent.*—To 52 mL 1.11M KH_2PO_4 (J.T. Baker, Phillipsburg, NJ, analyzed reagent grade), add 25 mL 1.54M TBAH solution to prepare 77 mL 0.5M tetra-*n*-butylammonium phosphate (TBAP), pH 7.2. Filter TBAP reagent through Millipore apparatus, and store in a capped brown bottle in dark when not in use.

(g) *Eluant.*—Mobile phase (degassed) consists of methanol (Baker)–water (60 + 40) containing 0.005M TBAP. Filter mobile phase through Millipore filter before use.

(h) *Reference compounds.*—Commercial samples of Amaranth (Williams (Hounslow) Ltd,

Middlesex, UK), Sunset Yellow FCF (FD&C Yellow No. 6, Williams (Hounslow) Ltd), and Tartrazine (FD&C Yellow No. 5, Butterfield Labs, Norfolk, UK) were used as received. The intermediates naphthionic acid (Matheson, East Rutherford, NJ), sulfanilic acid (Kleurstof Industries, Amersfoort, The Netherlands), Schaeffer's salt (2-naphthol-6-sulfonic acid, sodium salt, Allied Chemical, Buffalo, NY), R-salt (2-naphthol-3,6-disulfonic acid, sodium salt, Eastman-Kodak, Rochester, NY), and PyT (1-(4-sulfophenyl)-3-carboxy-5-hydroxypyrazolone, Williams (Hounslow) Ltd) were all purified with decolorizing carbon followed by recrystallization from ethanol. Purities of the standards were determined by spectrophotometry using published absorptivity values (18), and were greater than 92% for all intermediates.

Calibration Curves

Accurately weigh ca 0.02 g intermediate and transfer to 100 mL beaker. Dilute to ca 50 mL with water to dissolve intermediate. Add 1% NaOH, dropwise, and adjust pH of solution to 7.0. Transfer solution to 250 mL volumetric flask and dilute to volume with distilled, filtered water.

Into separate 100 mL volumetric flasks transfer 2 mL of each intermediate in a combination of (*1*) naphthionic acid and R-salt, (2) sulfanilic acid and Schaeffer's salt, and (3) sulfanilic acid and PyT. Add 1.0 mL 0.5M TBAP ion-pair reagent to each flask, and dilute to volume with distilled, filtered water. Mix thoroughly. Repeat this procedure using 5 mL of each intermediate.

Inject, separately, 5, 10, 15, and 20 μL of each solution, obtain chromatograms, and construct calibration curves of peak areas vs quantity of intermediate. Eight or more calibration points from duplicate injections are obtained for each intermediate.

Recovery Studies

Prepare spiked samples in the range 0.01–0.5% of each intermediate by adding appropriate volumes of combined intermediate solutions (above) to prepared aqueous solutions of each food color (containing 0.005M TBAP) adjusted to pH 7.0. Determine each intermediate combination in the food color at 5 different levels including the specification limit of 0.5% total intermediate concentration. (Analyze each food color before spiking to determine background levels of intermediates, and make appropriate corrections to recoveries obtained.)

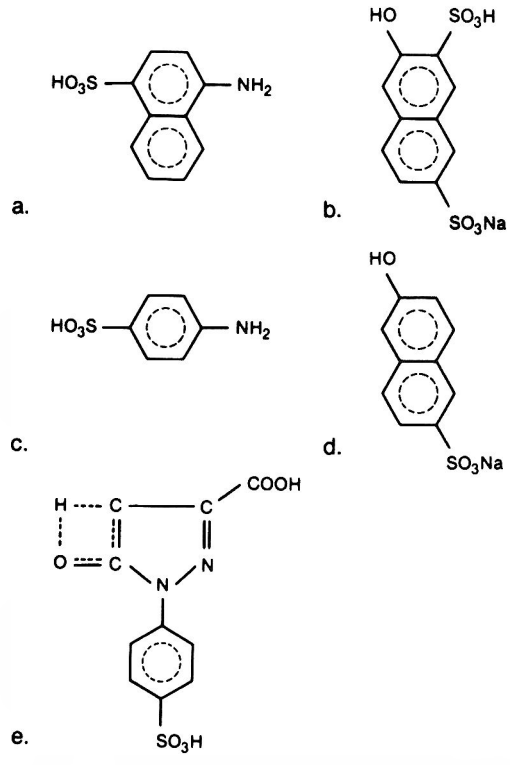

Figure 1. Structures of food color intermediates: (a) naphthionic acid; (b) R-salt; (c) sulfanilic acid; (d) Schaeffer's salt; (e) PyT.

Analysis of Commercial Samples

Accurately weigh ca 0.15 g dye and quantitatively transfer to 100 mL volumetric flask. Pipet 1.0 mL 0.5M TBAP ion-pair reagent into flask and dilute to volume with distilled water. Mix thoroughly to dissolve and use for LC analysis.

Inject 20 μL sample and, using suitable detector sensitivity (0.01–0.2 AUFS depending on quantity injected), obtain chromatograms of primary dyes and intermediates. Identify intermediates by comparing retention times with known standards. Calculate quantity of intermediates by measuring the peak area and determining nanograms present from the calibration curve.

If an intermediate is contained in the dye at less than 0.015% or cannot be detected, it may be necessary to use the specific wavelength for the individual intermediate instead of the compromise wavelengths in (**a**) in order to increase sensitivity. These wavelengths are 320 nm for naphthionic acid, 235 nm for R-salt, 249 nm for sulfanilic acid, 232 nm for Schaeffer's salt, and 257 nm for PyT. However, the compromise

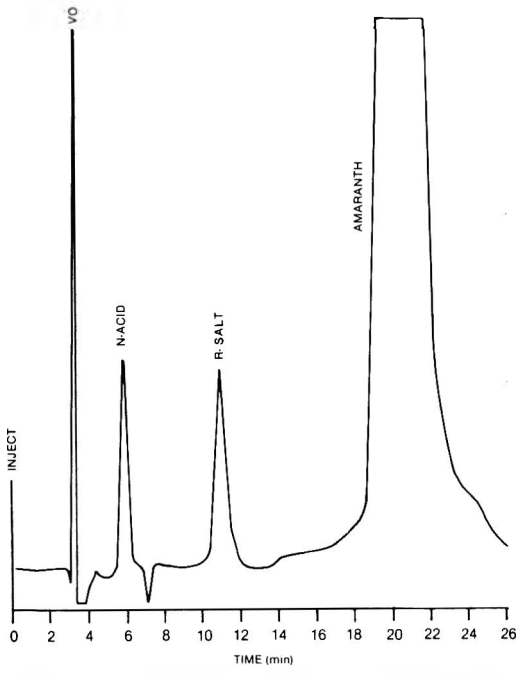

Figure 2. Chromatogram of commercial Amaranth spiked at 0.27% with naphthionic acid (N-acid) and at 0.27% with R-salt. Conditions as described in text. Wavelength 237 nm. Vo = column void volume.

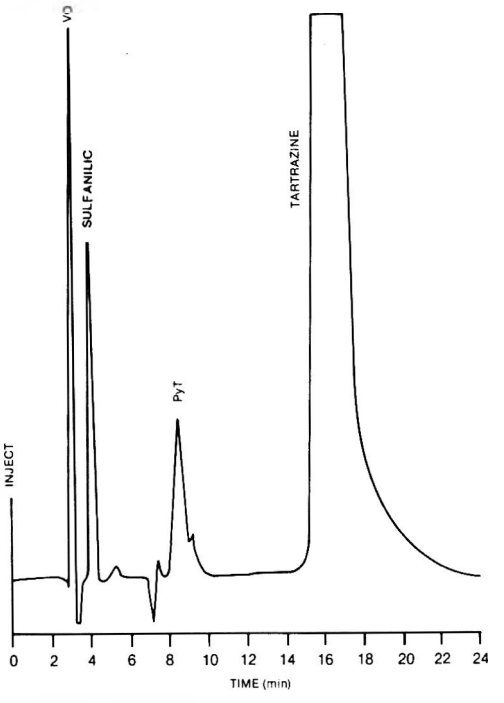

Figure 3. Chromatogram of commercial Tartrazine spiked at 0.28% with sulfanilic acid and at 0.18% with PyT. Conditions as described in text. Wavelength 249 nm. Vo = column void volume. The small unlabeled peaks near PyT and sulfanilic acid are unknowns.

wavelengths performed well for all commerical samples tested.

Results and Discussion

As shown in Figure 1, all intermediates studied contain at least one sulfonic acid group. It has been shown that ion pairing is particularly useful for the chromatographic separation of colors and subsidiaries containing sulfonic acid moieties (10, 17), so we thought that a similar chromatography system should be useful for the intermediates. This was in fact borne out in the results. The chromatographic system described performed well for all intermediates in the food colors examined. Figures 2–4 are typical chromatograms obtained from spiked color samples. Under the experimental conditions used, the intermediates were well resolved from the primary color peak and from any subsidiary dyes that may have been present. Fast Red E, the major subsidiary of Amaranth, eluted well after Amaranth (>100 min) as did the major subsidiaries of Tartrazine, and thus do not appear in Figures 2 and 3. These subsidiaries also did not interfere with subsequent analyses; they were apparently strongly retained by the column or eluted as undiscernible broad bands. However, 1-*p*-sulfophenylazo-2-naphthol-3,6-disulfonic acid, trisodium salt ("Skyark"), a subsidiary of Sunset Yellow FCF, does elute before the primary color, but does not interfere in the detection of the intermediates (Figure 4). The negative peaks in Figures 2–4 result from differences in composition of the injected solutions and the mobile phase and did not affect results.

Linear calibration curves were obtained over the range of about 5–2000 ng per injection of intermediate. Triplicate injections of standards at 40 ng produced results which were within ±3% of the mean. Normally, curves were prepared in the range 5–100 ng per injection.

Recovery data for the intermediates (Tables 1–3) ranged from 96 to 105% on individual intermediates spiked over the range 0.012–0.54% (1.2–54 ng/10 μg) primary dye. Detection limits for the specified detector and chromatography conditions were about 3 ng per injection. Based on a 30 μg injection of primary dye, this suggests that the method is suitable for detection of less than one-fifteenth the permitted maximum level.

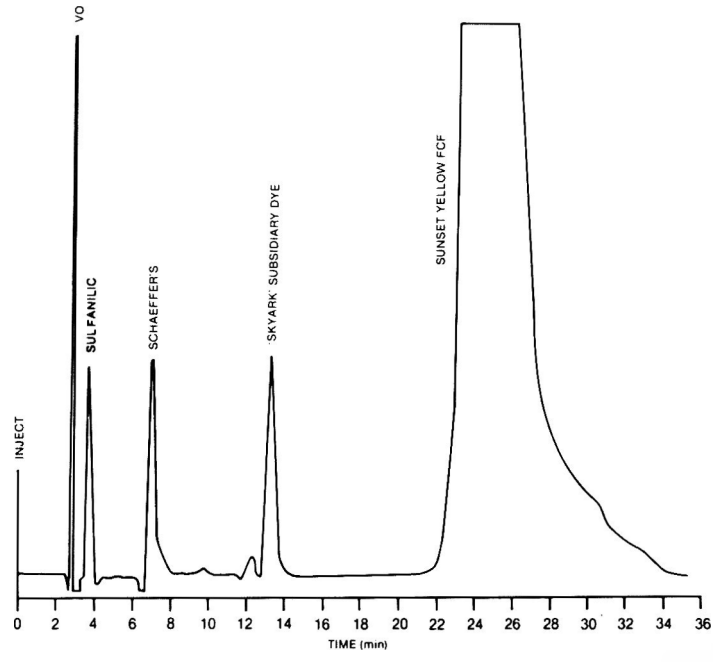

Figure 4. Chromatogram of commercial Sunset Yellow FCF spiked at 0.23% with sulfanilic acid and at 0.19% with Schaeffer's salt. Conditions as described in text. Wavelength 237 nm. Vo = column void volume. The small unlabeled peak near Skyark is unknown.

Table 1. Recovery of intermediates naphthionic acid (N-acid) and R-salt added to Amaranth

Added[a] (ng)		Recd[b] (ng)		Recd[b] (%)	
N-acid	R-salt	N-acid	R-salt	N-acid	R-salt
3.65	1.22	3.69	1.22	101	100
4.74	5.48	4.82	5.61	102	102
13.1	11.4	13.3	11.5	102	101
27.3	26.8	27.0	26.7	99.1	99.1
50.8	52.5	49.8	52.9	98.0	101

[a] Based on 10 μg Amaranth injected.
[b] Averages of duplicate or triplicate injections.

Table 2. Recovery of intermediates sulfanilic acid and Schaeffer's salt added to Sunset Yellow FCF

Added[a] (ng)		Recd[b] (ng)		Recd[b] (%)	
Sulf.	Sch.	Sulf.	Sch.	Sulf.	Sch.
2.65	2.35	2.57	2.32	97.2	100
4.79	4.38	4.70	4.38	98.1	100
10.2	7.50	9.81	7.44	96.2	99.1
22.5	18.6	22.1	18.6	98.0	100
43.0	37.1	43.0	36.1	100	97.3

[a] Based on 10 μg Sunset Yellow FCF injected.
[b] Averages of duplicate or triplicate injections.

Table 3. Recovery of intermediates sulfanilic acid and PyT added to Tartrazine

Added[a] (ng)		Recd[b] (ng)		Recd[b] (%)	
Sulf.	PyT	Sulf.	PyT	Sulf.	PyT
5.83	2.09	5.76	2.17	98.7	104
6.03	4.15	5.94	4.37	98.6	105
13.2	7.10	13.1	7.29	99.1	103
28.4	17.8	28.6	17.1	101	96.3
53.6	35.5	53.5	35.0	99.7	98.4

[a] Based on 10 μg Tartrazine injected.
[b] Averages of duplicate or triplicate injections.

Table 4. Total intermediate content[a] of some commercial dyes

Sample		Manufacturer (%)	This method (%)
Amaranth	A	0.17	0.20
	B	0.04	0.05
	C	0.08	0.12
Sunset Yellow FCF	A	0.03	0.024
	B	0.1	0.07
	C	<0.2	0.087
Tartrazine	A	<0.2	0.024
	B	0.22	0.18
	C	<0.05	0.01

[a] N-acid and R-salt in Amaranth; sulfanilic acid and Schaeffer's salt in Sunset Yellow FCF; sulfanilic acid and PyT in Tartrazine.

If improved detection limits are desired, detector sensitivity could be increased and, instead of using the compromise wavelengths, the specific wavelength for each intermediate could be used. For our purposes, the minimum detectable levels mentioned are more than adequate for monitoring food colors for intemediates at the permitted maximum limits.

Table 4 compares results obtained by this ion-pair liquid chromatographic technique for 9 commercial samples with those obtained by the manufacturer using ion-exchange liquid chromatography for all samples except Amaranth samples B and C where the cellulose column/ammonium sulfate procedure was used. It can be seen that results for the method described here are in good agreement.

This technique is particularly useful because samples of the 3 colors can be analyzed for intermediates, subsidiaries, and primary colors in either the same chromatographic system or with a minor change in the mobile phase for late eluting subsidiaries.

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Liquid Chromatographic Determination of 2,4-Dinitro-1-Naphthol and 1-Naphthol in External D&C Yellow No. 7

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A sensitive, reproducible method that uses liquid chromatography in the reverse phase mode with a C-18 column is described for determining 2,4-dinitro-1-naphthol and 1-naphthol in Ext. D&C Yellow No. 7. With this method, these 2 compounds are eluted in a reproducible pattern by increasing the organic nature of a buffered mobile phase. Average recoveries of 2,4-dinitro-1-naphthol and 1-naphthol added to samples of Ext. D&C Yellow No. 7 at levels ranging from 0.003 to 0.20% were 103.9 and 94.7%, respectively.

External D&C Yellow No. 7 (Colour Index No. 10316) is a synthetic color additive that is prepared by the nitration of the di- or trisulfonic acid of 1-naphthol. Every batch of certifiable Ext. D&C Yellow No. 7 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 externally applied drugs and/or cosmetics in the United States. The Code of Federal Regulations (CFR) (1) limits the amounts of 2,4-dinitro-1-naphthol and 1-naphthol to 0.03 and 0.2%, respectively, by weight of Ext. D&C Yellow No. 7.

At the present time, 1-naphthol is determined by a cellulose column/ammonium sulfate eluant procedure.[1] To determine 2,4-dinitro-1-naphthol in Ext. D&C Yellow No. 7, a petroleum ether extraction procedure (2) is used. Both of these methods give reproducible results but are time consuming. A reverse phase liquid chromatographic (LC) method using a C-18 column has been developed to separate 1-naphthol and 2,4-dinitro-1-naphthol from Ext. D&C Yellow No. 7. This method is faster than the currently used methods, is automated, and quantitates both compounds in the same analysis.

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 1.5 mL/min; column temperature ambient. Gradient program: from 30 to 55% B in 10 min; from 55 to 85% B in 20 min; from 85 to 100% B in 0.1 min; hold at 100% B for 5 min; from 100 to 30% B in 0.1 min; hold at 30% B for 5 min; then start injection program. Injection program: Signal is sent to auto-injector to rinse loop with water; then sample is injected. After injection, gradient program is started.

(b) *Detectors.*—Waters Model 440 dual wavelength detector (Waters Associates, Inc., Milford, MA 01757) operated at the following wavelengths and attenuations: 280 nm at 0.02 AUFS and 436 nm at 0.02 AUFS; or equivalent instrumentation.

(c) *Injector.*—Micromeritics Model 725 auto-injector equipped with a 50 μL loop (Micromeritics Instrument Corp., Norcross, GA 30093), or equivalent.

(d) *Recorder.*—Soltec Model 3314 (Soltec Corp., Sun Valley, CA 91352), or equivalent.

(e) *Chromatographic column.*—Whatman Partisil ODS-3 C-18 column, 25 cm × 4.6 mm id (Whatman Inc., Clifton, NJ 07014), or equivalent.

Reagents

(a) *Water.*—Deionized, distilled, and passed through Milli-Q water purification system (Millipore Corp., Bedford, MA 01730).

(b) *Primary solvent A.*—0.2M ammonium acetate. Weigh 15.42 g ammonium acetate and dissolve in 500 mL water. Transfer solution to 1 L volumetric flask, dilute to volume, and filter through 0.2 μm filter.

(c) *Secondary solvent B.*—Methanol, HPLC grade.

(d) *Stock solutions for calibration.*—(1) *1-Naphthol.*—Dissolve 10.0–11.0 mg in water in 200 mL volumetric flask, add ca 10 mg $Na_2SO_3.7H_2O$ as an antioxidant, and dilute to volume. Determine exact concentration of 1-naphthol stock solution from its UV spectrum; pipet 4 mL aliquot into 100 mL volumetric flask containing 25 g ammonium sulfate and 70 mL water and dilute to volume. Approximate absorptivity is 0.235 (L/(mg × cm)) at 228 nm. (2) *2,4-Dinitro-1-naphthol.*—Dis-

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[1] A copy of the 1-naphthol method can be obtained from the Division of Color Technology, 200 C St., SW, HFF-434, Washington, DC 20204.

solve 1.0–2.0 mg in water in 200 mL volumetric flask and dilute to volume. Determine exact concentration of 2,4-dinitro-1-naphthol stock solution from its visible spectrum. The molar absorptivity is 63.4 sq. cm/mole at 436 nm.

Calibration

Construct calibration curves for 1-naphthol and 2,4-dinitro-1-naphthol by plotting detector response (peak height) at 280 nm vs % by weight of the corresponding compound relative to the amount of Ext. D&C Yellow No. 7 in the solution. Prepare solutions for calibration as follows: Dissolve 200 mg Ext. D&C Yellow No. 7 (shown by previous analysis to be free of 1-naphthol and 2,4-dinitro-1-naphthol) in water and transfer to 100 mL volumetric flask. Add appropriate aliquots (0.5–10 mL) of 1-naphthol and 2,4-dinitro-1-naphthol stock solution to the volumetric flask and bring to volume. Analyze 6 calibration solutions containing fairly evenly spaced concentrations of each compound. For each compound in the solution, calculate C, % by weight relative to the amount of Ext. D&C Yellow No. 7 in the solution:

$C = V \times C' \times 100(\%) \times (1/200\ \text{mg})$ where V = volume of stock solution aliquot (mL), and C' = concentration of stock solution (mg/mL) determined spectrophotometrically.

On the basis of current allowable limits, the concentration of 1-naphthol, expressed as % by weight of Ext. D&C Yellow No. 7, should cover the range 0.01–0.25%; the range for 2,4-dinitro-1-naphthol concentrations should be 0.002–0.035%.

Mathematically choose best fitting straight line for calibration data by method of least squares. For each compound, calculate regression line, $y = bx + a$, by using following equations:

$$b = [\sum(x - \bar{x})(y - \bar{y})]/[\sum(x - \bar{x})^2]$$
$$= [\sum(xy) - [(\sum x \sum y)/n]]/[\sum x^2 - [(\sum x)^2/n]]$$
$$a = \bar{y} - b\bar{x}$$

where x = concentration of calibration standard; y = peak height response to calibration standard for component of interest; n = number of calibration solutions; b = slope of regression line; a = y intercept of regression line.

Determine linear correlation between peak height and concentration of standards by calculating correlation coefficient r:

$$r = [\sum(x - \bar{x})(y - \bar{y})]/\sqrt{[\sum(x - \bar{x})^2][\sum(y - \bar{y})^2]}$$

Value of r should be between 0.95 and 1.00.

Determination

Dissolve 200 mg sample of Ext. D&C Yellow No. 7 in 30 mL water. Transfer quantitatively to 100 mL volumeric flask, dilute to volume, and mix well. As described under *Calibration*, prepare standard solution of Ext. D&C Yellow No.7 containing both components of interest. Load autosampler in following way: first position, rinse vial which contains water; second position, blank gradient vial which contains water; third position, rinse vial; fourth position, Ext. D&C Yellow No. 7 standard; from this position on, all odd-numbered positions contain rinse vials and even-numbered positions contain sample vials.

Last sample is Ext. D&C Yellow No. 7 standard. If manual injector is to be used, prepare each solution just before injection and run samples in same order as above.

After all samples are loaded in auto-injector, start instrument. Place inlet tubes from pumps into appropriate solvents, which have been freshly prepared. Pump solvent through lines with column bypass valve opened. Pump long enough to ensure that all lines contain fresh solvent. Set pumps to 30% B at 2 mL/min for 10 min; then set flow to 0 and close bypass valve. Set flow to 1.5 mL/min and 30% B; 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 compound of interest in Ext. D&C Yellow No.7 sample, by substituting value of y (peak height for each compound of interest) and solving for x.

Results and Discussion

1-Naphthol absorbs only in the UV region, but 2,4-dinitro-1-naphthol has absorption at 280 and 436 nm; however, the 280 nm peak is used for the quantitation of both compounds. The 2,4-dinitro-1-naphthol peak at 436 nm provides additional qualitative information that is useful when only 1 of these compounds is present in a sample of Ext. D&C Yellow No. 7.

For the development of the method, we used concentrations of 1-naphthol and 2,4-dinitro-1-naphthol in the 9 calibration solutions that

Table 1. Recovery data and paired-difference 99% confidence interval results for Altex liquid chromatograph

Detn	1-Naphthol, %			2,4-Dinitro-1-naphthol, %		
	Added	Found	Rec.	Added	Found	Rec.
1	0.042	0.0395	94.0	0.03005	0.0298	99.2
2	0.084	0.0800	95.2	0.02404	0.0241	100.2
3	0.126	0.1209	96.0	0.01803	0.0182	100.9
4	0.168	0.1567	93.7	0.01202	0.0123	102.3
5	0.210	0.2028	96.6	0.00601	0.0064	106.5
6	0.021	0.0209	99.5	0.0150	0.0150	100.0
7	0.063	0.0577	91.6	0.009015	0.0095	105.4
8	0.105	0.0957	91.1	0.0030	0.0035	116.7
Av. Recovery			94.7			103.9
Paired-difference 99% confidence interval		0.0056 ± 0.0713			−0.0001 ± 0.0407	

covered the following ranges expressed as % by weight of Ext. D&C Yellow No. 7: 1-naphthol, 0.0105–0.210%; and 2,4-dinitro-1-naphthol, 0.00150–0.0301%.

The following limits of detection were calculated according to the method of statistical analysis described by Bailey et al. (3): 1-naphthol, 0.014%; and 2,4-dinitro-1-naphthol, 0.0020%.

Recovery studies with the method gave the following recovery ranges and averages for each compound of interest: 1-naphthol, 91.1–99.5%, 94.7%; and 2,4-dinitro-1-naphthol, 99.2–116.7%, 103.9%. Recovery data for individual determinations are given in Table 1. The paired-difference 99% confidence intervals all included zero.

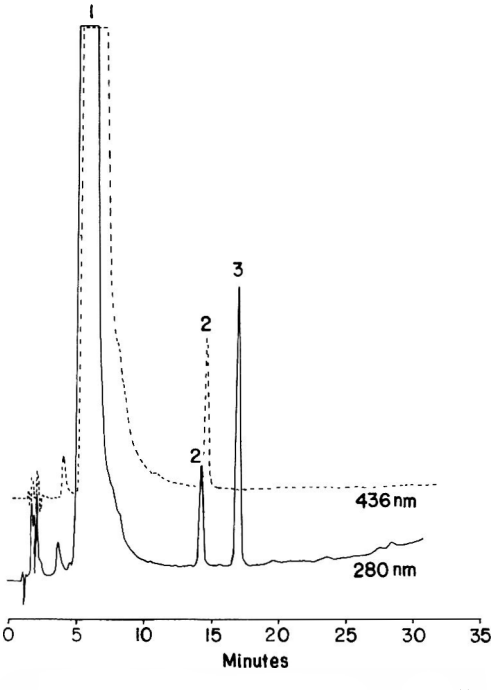

Figure 1. LC chromatogram of Ext. D&C Yellow No. 7 (peak 1). 2 = 2,4-dinitro-1-naphthol; 3 = 1-naphthol.

Table 2. Statistical analysis of results obtained with Varian liquid chromatograph

Parameter	1-Naphthol, %	2,4-Dinitro-1-naphthol, %
Limit of detection	0.022	0.0039
Recovery average	99.3	101.3
Recovery range	98.7–104.8	90.9–118.6
Paired-difference 99% confidence interval	0.003 ± 0.099	0.00009 ± 0.03

A new standard solution should be analyzed every day that Ext. D&C Yellow No. 7 samples are run. 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 with a corresponding correlation coefficient of less than 0.95 should be restandardized with new standards. If restandardization does not bring the correlation coefficient to within the acceptable range, the column should not be used.

Figure 1 shows the separation of 1-naphthol and 2,4-dinitro-1-naphthol from Ext. D&C Yellow No. 7. Because C-18 columns vary from company to company, modifications in the gradient may be needed to achieve similar results.

To verify that the results of the method could be duplicated, analyses were performed on a Varian 5060 liquid chromatograph with a Vista 401 data system. The column used was a Varian MCH-10 C-18. A summary of the results is shown in Table 2. The results are similar to

those obtained for the Altex liquid chromatograph.

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DRUGS

Simultaneous Spectrophotometric Determination of Amodiaquine-Primaquine Mixtures in Dosage Forms

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Using 0.1N hydrochloric acid as solvent, mixtures of amodiaquine and primaquine have been measured at 282 and 342 nm. The concentration of each can then be calculated by solving 2 simultaneous equations. Excellent recoveries from authentic samples were obtained and the method proved suitable for routine analysis.

Mixtures of amodiaquine and primaquine are sometimes dispensed for antimalarial therapy (1). The literature does not refer to any analytical method dealing with such a mixture. As a single component, amodiaquine could be determined using gravimetric (2), titrimetric (3), colorimetric, and atomic absorption spectrophotometric techniques (4). However, primaquine is also measured by all these reactions, so its presence would interfere with the results. Apart from the colorimetric methods published for the determination of primaquine (5, 6), the classical diazotization method (7) is applicable to macro amounts only.

The present report describes a simple spectrophotometric procedure that can be easily adapted in drug control laboratories for the simultaneous determination of both compounds.

Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Perkin Elmer 550 S.
(b) *Hydrochloric acid.*—0.1N (approximate).
(c) *Amodiaquine HCl.*—Chemically pure.
(d) *Primaquine phosphate.*—Chemically pure.

Both amodiaquine and primaquine salts must pass BP 1973 requirements.

Procedure

Accurately weigh an amount of crushed tablets containing primaquine phosphate and the equivalent of ca 150 mg amodiaquine HCl into 100 mL volumetric flask. Add 50 mL 0.1N HCl and shake 5 min to dissolve drugs. Dilute to volume with the same solvent. Filter, transfer 1 mL clear filtrate to another 10 mL volumetric flask, and dilute to volume with 0.1N hydrochloric acid. Measure absorbance of the latter solution at 282 and 342 against hydrochloric acid as a blank. Calculate concentration of both compounds, using the following equations:

$$\text{mg Primaquine phosphate} = 307\,A_{282} - 103\,A_{342} \quad (1)$$

$$\text{mg Amodiaquine HCl} = 273\,A_{342} - 21.3\,A_{282} \quad (2)$$

Results and Discussion

For the analysis of mixtures containing 2 absorbing substances, Vierordt's method (8) is usually applied. In these instances, it is necessary to select 2 points on the wavelength scale where the ratios of absorptivities are maxima. Referring to Figure 1, the most suitable wavelengths to be chosen for amodiaquine-primaquine mixtures would be 342 and 282 nm, respectively. Primaquine shows another peak at 266 nm, but this peak is unsuitable for primaquine analysis in the mixture because it coincides with a sharply sloping part of the amodiaquine spectrum. The figures stated in Equations 1 and 2 were obtained by numerical substitution for the values of Equations 3 and 4 below, taking into consideration the dilution factor in the procedure.

$$\text{Concn of primaquine phosphate} = (A_1\beta_2 - A_2\beta_1)/(\alpha_1\beta_2 - \alpha_2\beta_1) \quad (3)$$

$$\text{Concn of amodiaquine HCl} = (A_2\alpha_1 - A_1\alpha_2)/(\alpha_1\beta_2 - \alpha_2\beta_1) \quad (4)$$

where A_1 and A_2 are absorbance values of the mixture at 282 and 342 nm, respectively; α and β represent the absorptivity values of primaquine phosphate and amodiaquine HCl, respectively, at the relevant wavelengths.

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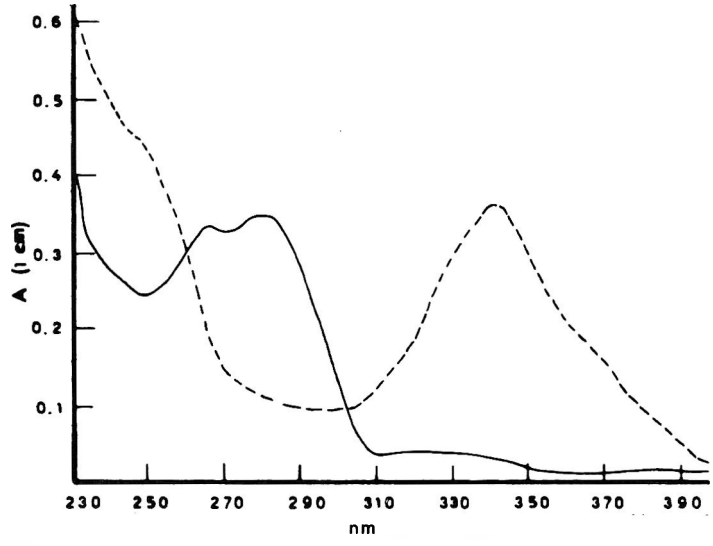

Figure 1. Absorption spectra of amodiaquine HCl (---) and primaquine phosphate (—) (1 mg/100 mL of each in 0.1N HCl).

Table 1. Recovery from authentic samples of amodiaquine hydrochloride and primaquine phosphate at different ratios

Ratio amod.: prim.	No. of exp.[a]	Concentration range, mg/100 mL		Mean recoveries, % (±SD)[b]	
		Amod. HCl	Prim. phos.	Amod. HCl	Prim. phos.
15:1	7	1.2–3.0	0.08–0.2	100.06 ± 0.3	89.25 ± 1.79
15:1.5	7	1.2–3.0	0.12–0.3	100.44 ± 0.3	97.04 ± 1.73
15:2	9	0.6–3.0	0.08–0.4	101.36 ± 0.75	100.24 ± 1.1
15:2.5	8	0.6–3.0	0.10–0.5	100.45 ± 0.83	99.18 ± 0.96
15:3	9	0.6–3.0	0.12–0.8	99.42 ± 0.53	99.17 ± 1.95
15:3.5	9	0.6–3.0	0.14–0.7	99.2 ± 0.89	99.86 ± 1.33
15:4	9	0.6–3.0	0.16–0.8	98.38 ± 0.28	100.09 ± 0.64
15:5	9	0.6–3.0	0.2–1.0	99.11 ± 0.39	99.51 ± 0.69

[a] Each experiment is the average of at least 2 determinations.
[b] $P = 0.05$.

Table 2. Recovery from authentic samples of amodiaquine hydrochloride and primaquine phosphate admixed in medicinally recommended ratio

Exp.[a]	Added, mg/100 mL		Recovery, %	
	Amod. HCl	Prim. phos.	Amod. HCl	Prim. phos.
1	1.2	0.2112	99.7	98.2
2	1.5	0.2640	100.4	98.8
3	1.8	0.3168	100.5	99.4
4	2.1	0.3695	100.4	100.3
5	2.4	0.4224	101.5	101.3
Mean			100.5	99.6
SD[b]			±0.8	±1.52

[a] Each experiment is the average of 3 determinations.
[b] $P = 0.05$.

The medicinally recommended ratio of amodiaquine hydrochloride to primaquine phosphate is 150:26.4 (about 6:1) (1). It is therefore necessary to determine the ratios within which primaquine can be accurately determined in the presence of excess amodiaquine. Table 1 shows that at the ratios 1:15 and 1.5:15 (primaquine phosphate:amodiaquine hydrochloride), a large error occurs in the determination of primaquine content. This may be attributed to overlapping of the spectrum of the latter by that of amodiaquine hydrochloride. However, within ratios starting from 1:7.5 primaquine phosphate to amodiaquine hydrochloride, recoveries of primaquine are good. Consequently, the medici-

Table 3. Application of proposed spectrophotometric method to simultaneous determination of amodiaquine hydrochloride and primaquine phosphate in a pharmaceutical preparation [a]

Recovery of std addns [b]			
Amod. HCl		Prim. phos.	
Added, mg/tab.	Rec., %	Added, mg/tab.	Rec., %
20	101.36	—	—
30	100.93	—	—
50	100.1	—	—
	—	5	99.96
	—	10	100.00
	—	15	100.10

[a] Camoprima tablets: Declared: 150 mg amodiaquine HCl + 26.4 mg primaquine phosphate/tablet; found 150 mg + 26.22 mg/tablet, respectively.
[b] $P = 0.05$.

nally recommended ratio, 1:6 primaquine phosphate to amodiaquine hydrochloride, can be safely determined by using the proposed method. The results obtained in Tables 2 and 3 augment these findings.

On the other hand, trials made to adapt pH-induced difference spectrophotometry depending on the phenolic nature of amodiaquine and the nonphenolic nature of primaquine failed to produce a suitable analytical procedure. This is mainly caused by the fact that solutions of amodiaquine hydrochloride in alkaline media are unstable.

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Liquid Chromatographic Determination of Methyldopa and Methyldopa-Thiazide Combinations in Dosage Forms

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A liquid chromatographic (LC) method, using a reverse phase C_{18} column, an acetic acid–methanol–water mobile phase, and detection at 280 nm, was developed for the determination of methyldopa in tablets and oral suspensions and combinations of methyldopa with hydrochlorothiazide or chlorothiazide in tablets. A mixture of these 3 drugs was resolved in <8 min. Detector responses were linear for the following amounts (mg/mL) of drug injected: methyldopa 0.031–0.393, chlorothiazide 0.019–0.114, and hydrochlorothiazide 0.004–0.083. Recoveries from commercial dosage forms ranged from 99.1 to 100.9% for methyldopa, 99.2–100.4% for chlorothiazide, and 100.0–101.2% for hydrochlorothiazide. Replicate injections of methyldopa, chlorothiazide, and hydrochlorothiazide standard preparations alone or in combination gave overall relative standard deviations of <1.6% (n = 10). The results for methyldopa tablets by the proposed method were in agreement with those obtained by the USP XX method. The LC method detected as little as 0.6 μg 3-*O*-methylmethyldopa/mL and 0.5 μg 4-amino-6-chloro-1,3-benzenedisulfonamide/mL, which are sometimes found as contaminants of methyldopa and thiazides, respectively, and resolved methyldopa from its methyldopa glucose adduct, a substance found in methyldopa oral suspensions.

Methyldopa is used in the treatment of mild to moderate hypertension. In severe hypertensive states, or when the hypertension is accompanied by marked sodium and water retention, the drug is often used in combination with either chlorothiazide or hydrochlorothiazide (1). Methyldopa has been determined in dosage forms by fluorometry (2, 3), ultraviolet (UV) spectrophotometry (4), and colorimetry (5, 6). Either these methods are nonspecific or they require close control of certain experimental conditions such as temperature and pH. Chu (7) resolved commercial mixtures of methyldopa with thiazide diuretics, using ion exchange chromatographic separation and spectrophotometric determination of each analyte in the eluates. Although the method is specific, it is lengthy and time consuming. Watson and Lawrence (8) reported a gas-liquid chromatographic method for methyldopa in tablets and raw material; however, a lengthy derivatization step is required. More recently, several liquid chromatographic (LC) methods have been proposed for the determination of methyldopa in biological samples (9–12) and pharmaceuticals (13, 14). Using reverse phase chromatography and electrochemical detection, Freed and Asmus (9) and Kochak and Mason (10) determined methyldopa in plasma and tissues, respectively. Cooper et al. (11) measured methyldopa in serum by combining ion exchange chromatographic separation with electrochemical detection. Mell and Gustafson (12) determined free methyldopa in urine samples by reverse phase LC with UV detection, and reported no interference by hydrochlorothiazide. Honigberg et al. (13) proposed 2 types of chromatographic columns and several mobile phases for the separation of diuretic-antihypertensive mixtures, but in these systems, methyldopa showed tailing, multiple peaks, or incomplete resolution from the thiazide diuretics. Rao et al. (14) proposed an LC method for the determination of methyldopa and hydrochlorothiazide in combination dosage forms but no work was done on the impurities.

The present paper reports a rapid and accurate LC method for the determination and tentative identification of methyldopa and methyldopa with either hydrochlorothiazide or chlorothiazide combinations in dosage forms. The proposed method is also useful for the detection of 3-*O*-methylmethyldopa and methyldopa glucose adduct in methyldopa and of 4-amino-6-chloro-1,3-benzenedisulfonamide in hydrochlorothiazide and chlorothiazide.

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Experimental

Apparatus

(a) *Liquid chromatograph.*—Tracor Model 950 solvent pump, Model 970A variable wavelength detector, and Model TS-10 recorder (Tracor Instruments, Inc., Austin, TX 78721). Samples were injected through 20 μL Rheodyne Model 7125 injection loop (Rheodyne Inc., Berkeley, CA 94710).

(b) *Chromatographic column.*—μBondapak C_{18}, 30 cm × 3.9 mm id, 10 μm particle size (Waters Associates, Milford, MA 01757).

Operating conditions: column temperature ambient, mobile phase flow rate 1.5 mL/min, detector wavelength 280 nm, chart speed 1 cm/min.

(c) *Filters.*—Millipore type FH (pore size 0.5 μm) (Millipore Corp., Bedford, MA 01730).

Reagents

(a) *Solvents.*—LC or analytical reagent grade, used without further purification.

(b) *Mobile phase.*—3% Aqueous acetic acid–methanol (96 + 4). Filter through type FH filter.

(c) *Internal standard.*—Weigh accurately ca 110 mg Theobromine USP Reference Standard into 500 mL volumetric flask, add ca 400 mL mobile phase, warm on steam bath with occasional swirling, let flask and its contents cool to ambient temperature, dilute to volume with mobile phase, and mix. Filter solution with Whatman No. 40 or equivalent paper, discarding first 20 mL filtrate.

(d) *Methyldopa standard.*—USP Reference Standard. Determine water content by titrimetric method (15) before use.

(e) *Thiazide standard.*—Chlorothiazide USP Reference Standard or Hydrochlorothiazide USP Reference Standard. Dry at 105°C 1 h before use.

(f) *3-O-Methylmethyldopa standard.*—USP Reference Standard. For LC analysis, dissolve in methanol, and dilute aliquot with enough mobile phase to provide solution giving discernible peak response. As low as 2 μg/mL was detectable at sensitivity setting of 0.02 absorbance unit full scale (AUFS).

(g) *4-Amino-6-dichloro-1,3-benzenedisulfonamide.*—USP Reference Standard. For LC analysis, dissolve in methanol, and dilute aliquot with enough mobile phase to provide solution giving discernible peak response. As low as 1 μg/mL was detectable at sensitivity setting of 0.02 AUFS.

(h) *Methyldopa glucose adduct.*—Merck Sharp & Dohme Research Laboratories, Rahway, NJ. For LC analysis, dissolve in methanol and dilute aliquot with enough mobile phase to provide solution giving discernible peak response.

Sample Preparation

Methyldopa tablets and methyldopa-chlorothiazide tablets.—Weigh and finely powder ≥20 tablets. Transfer accurately weighed portion of powder, equivalent to ca 125 mg methyldopa, to 250 mL volumetric flask, add 150 mL methanol–water (1 + 1), and sonicate 15 min more than needed to completely dissolve corresponding standards. Dilute to volume with methanol–water (1 + 1), mix, and filter through type FH filter, discarding first 10 mL filtrate. Pipet 8.0 mL filtrate and 10.0 mL internal standard solution into 50 mL volumetric flask, mix, dilute to volume with mobile phase, and mix.

Methyldopa-hydrochlorothiazide tablets.—Follow procedure for methyldopa tablets except transfer 20 mL sample filtrate and 6.0 mL internal standard solution to 50 mL volumetric flask, instead of 8.0 and 10.0 mL, respectively.

Methyldopa oral suspension.—Transfer accurately measured volume methyldopa oral suspension, equivalent to ca 150 mg methyldopa, to 250 mL volumetric flask, dissolve and dilute to volume with water, mix, and filter, discarding first 10 mL filtrate. Pipet 8.0 mL filtrate and 10.0 mL internal standard solution into 50 mL volumetric flask, mix, dilute to volume with mobile phase, and mix.

Standard Preparation

Tablets.—Transfer accurately weighed sample methyldopa standard, equivalent to 50 mg drug on anhydrous basis, to 100 mL volumetric flask. Add accurately weighed amount corresponding thiazide diuretic standard so that ratio of methyldopa standard to diuretic standard is same as that declared for tablet. For example, take 50 mg methyldopa standard and 5 mg diuretic standard if tablet sample declares 250 mg methyldopa and 25 mg diuretic. To this mixture add 70 mL methanol–water (1 + 1) and sonicate to dissolve. Let flask and its contents cool to room temperature. Dilute to volume with methanol–water (1 + 1). Mix thoroughly and filter solution through type FH filter, discarding first 20 mL filtrate. Prepare standard solution for methyldopa-hydrochlorothiazide sample as follows: Pipet 10.0 mL filtrate and 3.0 mL internal standard solution into 25 mL volumetric flask, and mix. Dilute to volume with mobile phase, and mix. For all other samples, prepare standard solution as follows: Pipet 4.0 mL filtrate and 5.0 mL internal standard solution into 25 mL volumetric flask, dilute to volume with mobile phase, and mix.

Methyldopa oral suspension.—Accurately weigh ca 60 mg anhydrous Methyldopa USP Reference Standard and place in 100 mL volumetric flask. Dissolve and dilute to volume with water, mix, and filter through type FH filter, discarding first 10 mL filtrate. Pipet 8.0 mL filtrate and 10.0 mL

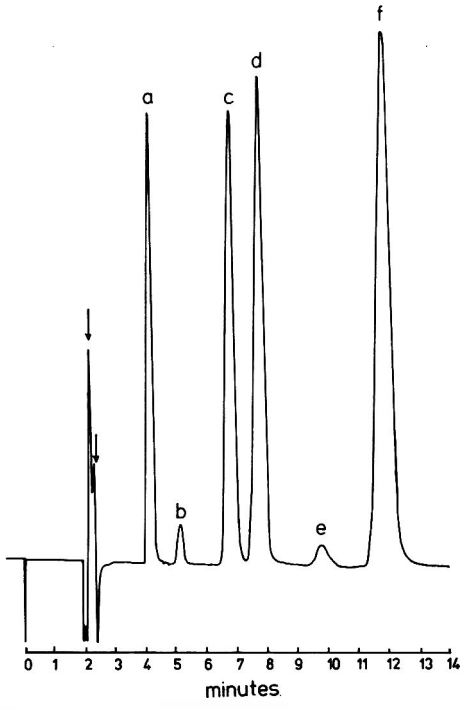
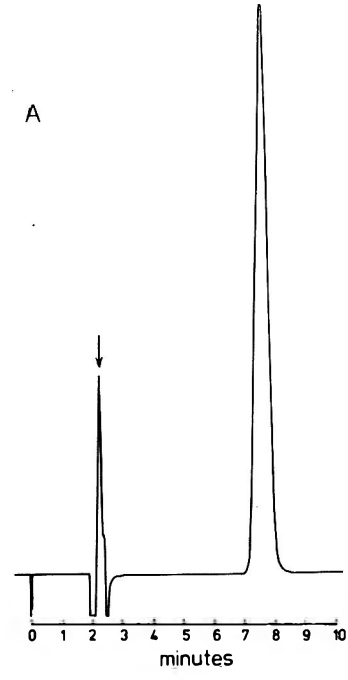


Figure 1. LC separation of a, methyldopa; b, 4-amino-6-chloro-1,3-benzenedisulfonamide; c, chlorothiazide; d, hydrochlorothiazide; e, 3-*O*-methylmethyldopa; and f, theobromine in a mixed standard solution. Detection wavelength, 280 nm. Arrows indicate solvent peaks. Column μBondapak C_{18}; mobile phase 3% aqueous acetic acid-methanol (96 + 4).

internal standard solution into 50 mL volumetric flask, mix, dilute to volume with mobile phase, and mix.

Determination

Equilibrate LC column with mobile phase at flow rate of 1.5 mL/min. Inject 20 μL corresponding standard preparation, and adjust sensitivity of detector so that peak responses are ca 35–95% of full scale. Change sensitivity of detector when going from peak of methyldopa to peak of hydrochlorothiazide. If necessary, make same adjustment for chlorothiazide. Volume of internal standard solution added to sample and



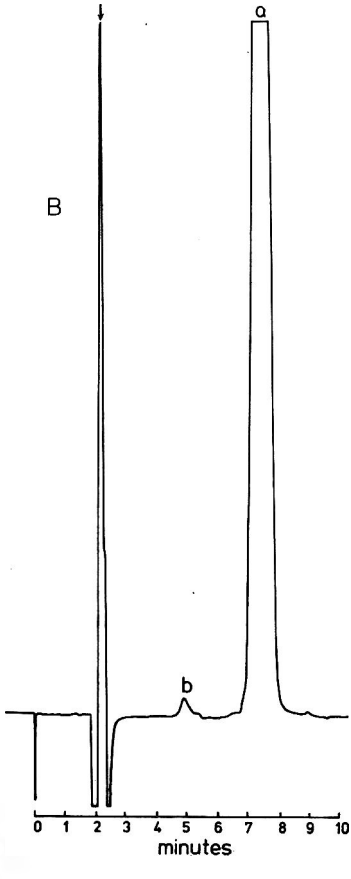


Figure 2. A, Chromatogram of hydrochlorothiazide in commercial tablet sample. Detector set at 280 nm and 0.16 AUFS. Arrow indicates solvent peak. B, Chromatogram of a, hydrochlorothiazide in commercial tablet sample spiked with b, 4-amino-6-chloro-1,3-benzenesulfonamide. Detector set at 280 nm and 0.04 AUFS. Arrow indicates solvent peak. Other chromatographic conditions as for Figure 1.

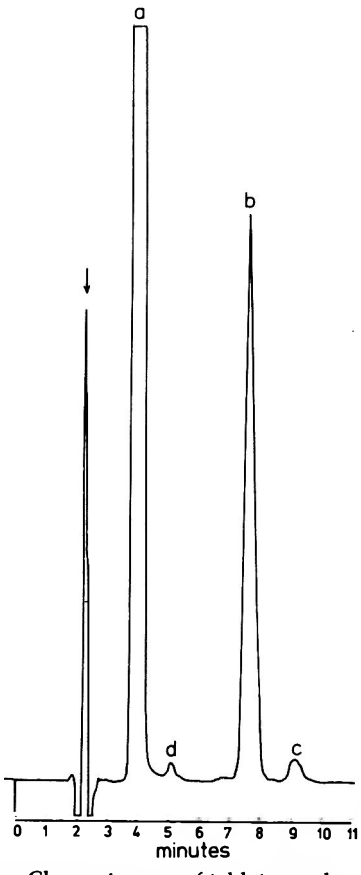


Figure 3. Chromatogram of tablet sample containing a, methyldopa and b, hydrochlorothiazide which was spiked with c, 4-amino-6-chloro-1,3-benzenesulfonamide, and d, 3-*O*-methylmethyldopa. Detector set at 280 nm and 0.04 AUFS. Arrow indicates solvent peaks. Other chromatographic conditions as for Figure 1.

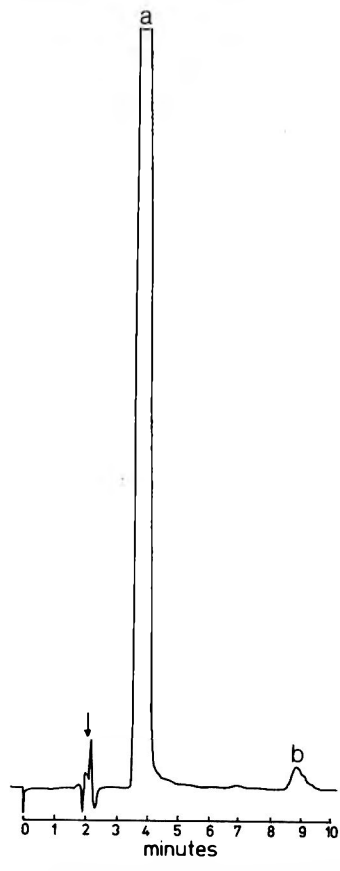


Figure 4. Chromatogram of tablet sample of a, methyldopa spiked with b, 3-*O*-methylmethyldopa. Detector set at 280 nm and 0.04 AUFS. Arrow indicates solvent peaks. Other chromatographic conditions as for Figure 1.

standard preparations can be adjusted to obtain appropriate peak height response for internal standard. Make replicate injections of each standard solution and compare peak height responses to ascertain reproducibility of system. In suitable system, relative standard deviation for triplicate injections is ≤2%. Proceed with analysis of sample, using 20 μL injections for each sample and standard solution. Methyldopa, chlorothiazide, and hydrochlorothiazide peaks elute in ca 4, 7, and 8 min, respectively. Retention time of theobromine, internal standard, should be ≥9 min. Resolution factor between methyldopa and chlorothiazide and between hydrochlorothiazide and theobromine should be >3.5.

Calculations

Calculate quantity of drug, using peak height response ratios (R and R') relative to internal standard, by following equations:

$$\text{Methyldopa or thiazide, mg/tablet} = (R/R') \times C \times (T/S) \times \text{sample diln}$$

$$\text{Methyldopa, mg/mL} = (R/R') \times C \times \text{sample diln/mL sample used}$$

where R and R' = peak height response ratios for sample and standard preparations relative to internal standard, respectively; C = concentration of standard preparation, mg/mL; T = average tablet weight, mg/tablet; and S = sample weight, mg.

Results and Discussion

Figure 1 illustrates a typical separation of the 3 drugs, along with their accompanying impurities and the internal standard. Under the experimental conditions, the retention times

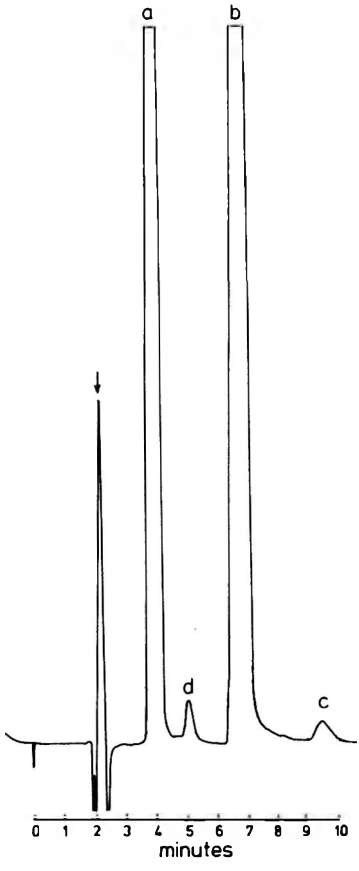


Figure 5. Chromatogram of tablet sample containing a, methyldopa and b, chlorothiazide, which was spiked with c, 4-amino-6-chloro-1,3-benzenedisulfonamide, and d, 3-*O*-methylmethyldopa. Detector set at 280 nm and 0.08 AUFS. Arrow indicates solvent peak. Other chromatographic conditions as for Figure 1.

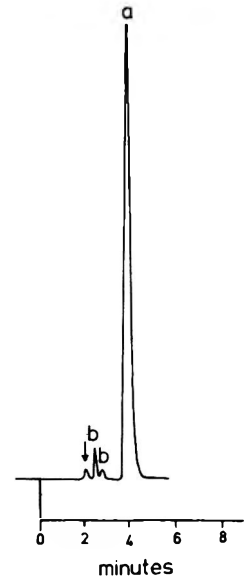


Figure 6. Chromatogram of oral suspension of a, methyldopa, spiked with b, methyldopa glucose adduct. Adduct is present as mixture of 2 isomers. Detector set at 280 nm and 0.16 AUFS. Arrow indicates solvent peak. Other chromatographic conditions as for Figure 1.

(min) were approximately: methyldopa 4; 4-amino-6-chloro-1,3-benzenedisulfonamide 5; chlorothiazide 6.8; hydrochlorothiazide 7.8; 3-*O*-methylmethyldopa 9.8, and theobromine, 11.8. Figures 2–5 illustrate the separation of the active ingredients in methyldopa and in methyldopa-thiazide commercial dosage forms from their related impurities. Figure 6 shows that the proposed method will separate methyldopa from methyldopa glucose adduct, a reaction product found in methyldopa oral suspensions. During the chromatographic separation of methyldopa from hydrochlorothiazide, the attenuation setting of the detector had to be changed because of the wide difference in detector responses between these 2 drugs due to differences in their corresponding concentrations in the dosage forms and/or absorptivity values.

Based on the determination of peak height responses of standards to internal standard, the proposed method was linear at the following ranges of amounts of substance injected: methyldopa, from 0.031 to 0.393 mg/mL; chlorothiazide, 0.019–0.114 mg/mL; hydrochlorothiazide, 0.004–0.083 mg/mL; 3-*O*-methylmethyldopa, 0.6–2.4 μg/mL; and 4-amino-6-chloro-1,3-benzenedisulfonamide, 0.5–1.96 μg/mL.

Two studies were conducted to test the reproducibility of the LC method. In one, 10 replicate aliquots of synthetic tablet formulations containing methyldopa, chlorothiazide, and hydrochlorothiazide were injected, and the peak height ratios of each drug to the internal standard were measured (Table 1). In the other study, each of 4 commercial dosage forms were assayed 6 times for drug content (Table 2).

Accuracy was determined by recovery studies of methyldopa, chlorothiazide, and hydrochlorothiazide added to commercial dosage forms (Table 3). The average recovery values (%) obtained were: methyldopa 99.75, chlorothiazide 99.8, and hydrochlorothiazide 100.6. The results of a comparison of the LC method for methyldopa with the USP XX method, using commercial dosage forms, are shown in Table 4. Results

Table 1. Results of reproducibility study on standards

Compound	Amount, mg	Ratio,[a,b] mean ± SD	Rel. SD, %
Methyldopa	125	1.34 ± 0.0064	0.47
Methyldopa + chlorothiazide	250 250	1.25 ± 0.0050 1.27 ± 0.0050	0.40 0.39
Methyldopa + hydrochlorothiazide	500 30	0.87 ± 0.0099 0.88 ± 0.0062	1.14 0.70

[a] Based on 10 replicate injections of each standard solution.
[b] Ratio of peak height of drug standard/peak height of internal standard.

Table 2. Results of reproducibility study on commercial dosage forms

Product	Amount declared, mg	Amount found, mean ±SD,[a]	Rel. SD, %
Methyldopa tablet	500	99.2 ± 0.68	0.69
Methyldopa + chlorothiazide tablet	250 250	105.4 ± 0.86 99.2 ± 0.51	0.82 0.52
Methyldopa + hydrochlorothiazide tablet	500 30	101.1 ± 0.66 99.7 ± 1.56	0.65 1.56
Methyldopa oral suspension	50[b]	102.3 ± 0.99	0.97

[a] Based on 6 replicate injections of each sample.
[b] mg/mL.

Table 3. Recovery of drugs added to commercial dosage forms

Dosage form	Declared, mg/tab.	Drug added[a]	Amt added, mg	Amt recd,[b] mg	Rec., %
Methyldopa tablets	500	Me-Dopa	26.82	26.59	99.1
Methyldopa tablets	125	Me-Dopa	26.48	26.26	99.2
Methyldopa + hydrochlorothiazide tablets	500 50	Me-Dopa HC	22.54 2.43	22.57 2.46	100.1 101.2
Methyldopa + hydrochlorothiazide tablets	500 30	Me-Dopa HC	22.54 1.51	22.74 1.51	100.9 100.0
Methyldopa + chlorothiazide tablets	250 250	Me-Dopa CH	22.72 24.40	22.57 24.49	99.3 100.4
Methyldopa + chlorothiazide tablets	250 150	Me-Dopa CH	25.60 15.60	25.58 15.48	99.9 99.2

[a] Me-Dopa = methyldopa; HC = hydrochlorothiazide; CH = chlorothiazide.
[b] Average of 2 determinations.

Table 4. Determination of methyldopa and methyldopa-thiazide combinations in commercial tablets

Sample[a]	Declared, mg/tab	Found, % of declared: Me-Dopa USP XX	Me-Dopa LC	HC LC	CH LC
Me-Dopa	150	97.8	97.4	—	—
Me-Dopa	250	99.5	99.5	—	—
Me-Dopa	500	100.6	98.4	—	—
Me-Dopa + HC	250 25	101.3 —	101.2 —	— 99.2	— —
Me-Dopa + HC	500 30	103.0 —	101.8 —	— 94.2	— —
Me-Dopa + CH	250 250	99.8 —	101.5 —	— —	— 100.5

[a] Me-Dopa = methyldopa; HC = hydrochlorothiazide; CH = chlorothiazide.

obtained by these 2 methods differed by <2%. Table 4 also shows the assay values for thiazide drugs using the proposed method.

The LC method eliminates the need to determine methyldopa and thiazide diuretics by separate methods or the requirement of a preliminary separation of the 2 types of drug. In addition, its sensitivity is such that it can be used as a purity-indicating test to ascertain the presence of as little as 1% of both 3-*O*-methylmethyldopa in methyldopa and 4-amino-6-chloro-1,3-benzenedisulfonamide in diuretics such as chlorothiazide and hydrochlorothiazide. The compendial limits (15) for 3-*O*-methylmethyldopa and 6-chloro-1,3-benzenedisulfonamide in raw materials are 0.5 and 1%, respectively. Raw material/samples of methyldopa, hydrochlorothiazide, and chlorothiazide were analyzed by the proposed method; each contained <0.3% of its corresponding impurity. Methyldopa glucose adduct was also detected in the methyldopa oral suspension.

In summary, the proposed method is rapid, selective, accurate, and precise. It gave results for methyldopa in tablets which were in excellent agreement with those obtained by the compendial method. A collaborative study of this method is currently in progress and the results will be reported.

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Separation and Characterization of Amine Drugs and Their Enantiomers by Capillary Column Gas Chromatography-Mass Spectrometry

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A gas chromatograph-mass spectrometer-data system equipped with a capillary column is used to analyze commonly abused amine drug mixtures. Enantiomeric amines are analyzed as *N*-trifluoroacetyl-*l*-prolyl chloride derivatives. The 10 compounds included in this study are amphetamine, methamphetamine, norephedrine, ephedrine, 3,-4-methylenedioxyamphetamine, *N,N*-dimethyltryptamine, *N,N*-diethyltryptamine, *N,N*-dimethyl-5-methoxytryptamine, mescaline, and caffeine. All compounds, including possible enantiomers, are resolved and identified by the described method.

Due to the frequent manufacture and abuse (1) of methamphetamine and related amine drugs, numerous studies concerning these drugs have been reported (2–12). Most of these studies were centered on the identification of impurities found in illicit methamphetamine and amphetamine preparations (2–7); others (8–10) were directed toward the separation and identification of these amine drugs.

Recently, we focused on the application of analytical methodology that could be used to determine amphetamine and methamphetamine enantiomers (11, 12). This effort was prompted by the consideration of differences in pharmacological effects and government regulatory measures (13, 14) of enantiomers. Furthermore, the analysis of enantiomeric composition in an illicit preparation may in some cases yield useful information regarding its origin of manufacture or distribution.

The application of gas chromatography to the separation of enantiomers with chiral derivatizing reagents has been well documented (15–17) and reviewed (18). *N*-Trifluoroacetyl-*l*-prolyl chloride (*l*-TPC) has been widely used for enantiomeric separation of amino acids and some amine drugs. (12, 15–17, 19). In general, these studies have focused on biochemical application, and have neglected the study of combination drug mixtures exhibiting optical activity. With the broad potential of finding these drugs in combination in samples such as "look-alike" drugs, the development of methods suitable for the characterization of individual drugs, drug mixtures, their enantiomers, and impurities will be useful.

Although a conventional mass spectrometer, used as a detector for a gas chromatograph, is incapable of differentiating enantiomers, it does facilitate the identification of other compounds. Furthermore, single ion chromatograms are useful in displaying inadequately separated peaks.

The study presented here applies a high efficiency capillary column gas chromatographic-mass spectrometric approach (12) to the analysis of mixtures of the following drugs and their possible enantiomers: amphetamine (I), methamphetamine (II), norephedrine (III), ephedrine (IV), 3,4-methylenedioxyamphetamine (MDA) (V), mescaline (VI), *N,N*-dimethyltryptamine (DMT) (VII), *N,N*-diethyltryptamine (DET) (VIII), and *N,N*-dimethyl-5-methoxytryptamine (DMMT) (IX). Drugs that may exist as enantiomeric pairs are analyzed as *l*-TPC derivatives. Caffeine (X) is used as an internal standard. Structures of these compounds and TPC-derivatized amphetamine are shown in Figure 1.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph-mass spectrometer-data system.*—Hewlett-Packard (Palo Alto, CA) HP-5985, and 30 m × 0.2 mm id SE-54 fused silica glass capillary column (Alltech, Deerfield, IL).

(b) *Amine drugs.*—Obtained as indicated: *d*- and *l*-amphetamine, norephedrine, *d*-norephedrine hydrochloride, *d*-ephedrine hydrochloride, DMT, DET, DMMT, and mescaline hydrochloride (Aldrich Chemical Co., Milwaukee, WI); *d,l*-methamphetamine hydrochloride (Sigma Chemical Co., St. Louis, MO); ephedrine sulfate (prescription); MDA (Criminalistics Division, Chicago Police Dept).

(c) *Caffeine.*—Fisher Scientific Co.

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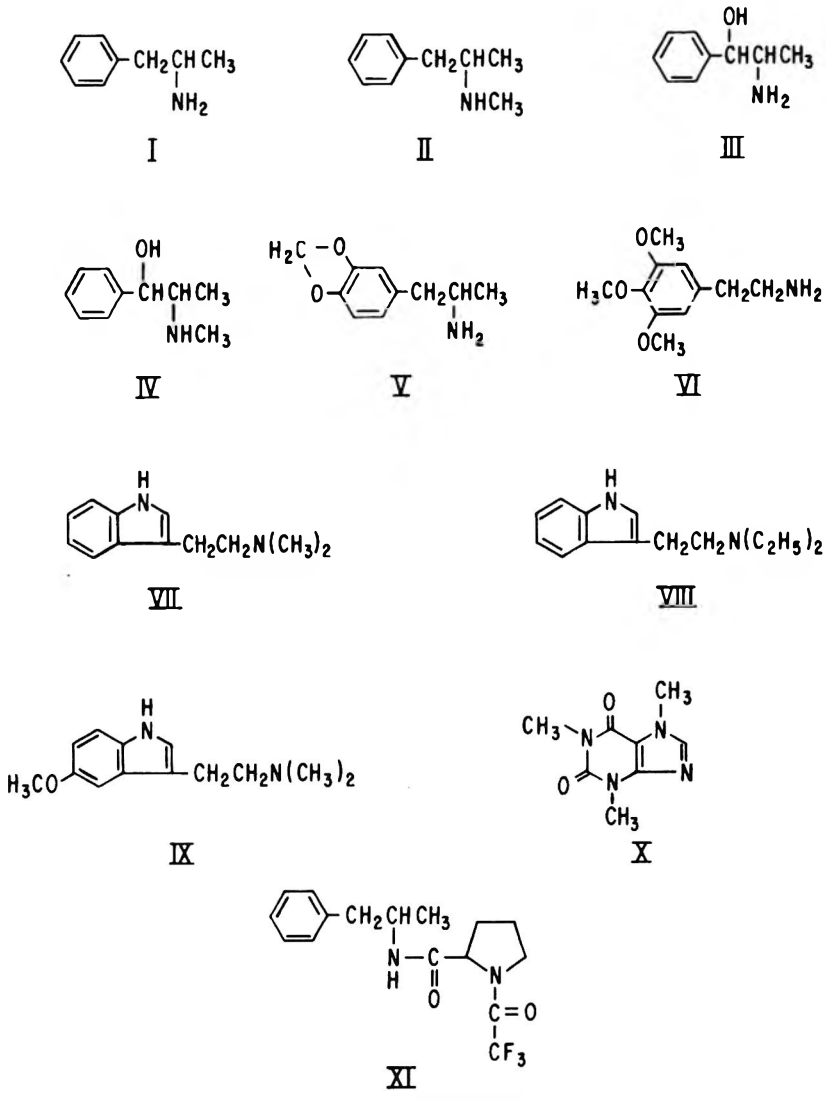

Figure 1. Structures of amphetamine (I), methamphetamine (II), norephedrine (III), ephedrine (IV), MDA (V), mescaline (VI), DMT (VII), DET (VIII), DMMT (IX), caffeine (X), and *N*-TFA-prolylamphetamine (XI).

(d) *l-TPC.*—(Regis Chemical Co., Morton Grove, IL) 0.1M in chloroform.

Procedure

Drugs in salt forms were dissolved in water, adjusted to alkaline, and then extracted with ether. Enantiomeric drugs were further converted to their *l*-TPC derivatives (*N*-TFA-*l*-prolyl-) by reacting with 0.1M *l*-TPC in chloroform, followed by cleaning and drying procedures (12). Caffeine, DMT, DET, DMMT, and mescaline were prepared as 5 mg/mL stock solutions, which were diluted 1:10 in methylene chloride before analysis.

Samples thus prepared were analyzed on the HP-5985 system. The mass spectrometer was operated in electron impact mode at 70 eV. The source temperature was maintained at 200°C. Mass units and their relative abundance were calibrated with perfluorotributylamine (20). Spectra were collected in the range m/z 45 to 500, and in most cases started at 15 min after injection. Gas chromatograph oven temperature programming conditions were as follows: 50°C for 1 min, then increasing 8°/min for 9 min, then 6°/min until reaching final temperature of 300°C. Conditions: injector temperature 250°C; chromatograph-mass spectrometer interface

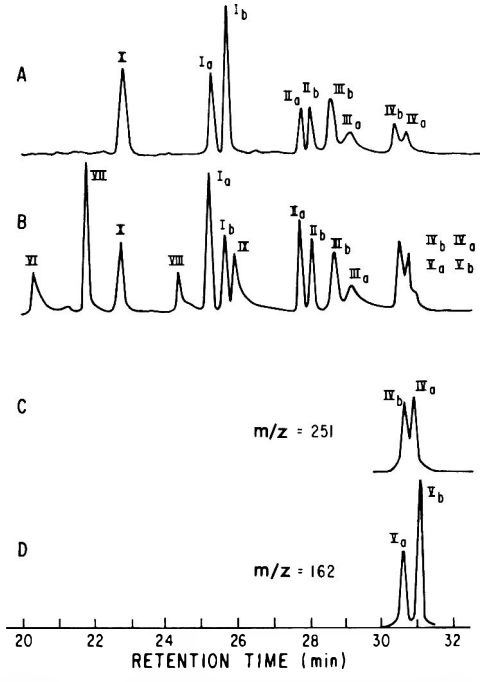

Figure 2. Total ion chromatograms of (A) a mixture containing caffeine (X), *N*-TFA-*l*-prolyl-*l*-amphetamine (I_a), *N*-TFA-*l*-prolyl-*d*-amphetamine (I_b), *N*-TFA-*l*-prolyl-*l*-methamphetamine (II_a), *N*-TFA-*l*-prolyl-*d*-methamphetamine (II_b), *N*-TFA-*l*-prolyl-*d*-norephedrine (III_b), *N*-TFA-*l*-prolyl-*l*-norephedrine (III_a), *N*-TFA-*l*-prolyl-*d*-ephedrine (IV_b), *N*-TFA-*l*-prolyl-*l*-ephedrine (IV_a); and (B) a mixture containing the compounds in (A) plus mescaline (VI), DMT (VII), DET (VIII), DMMT (IX), *N*-TFA-*l*-prolyl-*l*-MDA (V_a), *N*-TFA-*l*-prolyl-*d*-MDA (V_b). Single ion chromatograms of (C) compounds IV_b and IV_a (m/z 251); and (D) compounds V_a and V_b (m/z 162).

temperature 350°C; inlet pressure 15 psi; linear flow rate 40 cm/s. Helium was used as the carrier gas throughout all experiments.

Results and Discussion

Caffeine, amphetamine, methamphetamine, norephedrine, and ephedrine are commonly associated with look-alike drug products. All compounds, except caffeine, exist in enantiomeric pairs, and were derivatized with *l*-TPC. The total ion chromatogram of a mixture containing caffeine and *l*-TPC derivatives of the enantiomeric pairs is presented in Figure 2A.

Figure 2B is the total ion chromatogram of a mixture containing the compounds in Figure 2A and other common amine drugs in their free forms (mescaline, DMT, DET, and DMMT) or as *l*-TPC derivaives (MDA). The quantities of drug constituents in these chromatographic peaks range approximately from 200 to 600 ng.

With one notable exception, all compounds in Figure 2B are well resolved. Derivatives of ephedrine and MDA co-elute under the chromatographic conditions used. To clearly display the presence of these 2 pairs of compounds, single ion chromatograms for ephedrine (m/z 251) and MDA (m/z 162) derivatives are presented in Figures 2C and 2D, respectively. These chromatograms can be used for qualitative and quantitative analysis of these 2 compounds. However, as a result of inadequate resolution, quantitative determination of ephedrine enantiomeric composition may be difficult.

Major ions of each chromatographic peak in

Table 1. Chromatographic and mass spectrometric parameters

Compound No.	Compound	RRT[a]	Major mass fragments[b]
VI	mescaline	0.890	182(B), 167(51), 211(31), 151(15)
VII	DMT	0.960	58(B), 188(5), 130(4), 77(3)
X	caffeine	1.00	194(B), 109(63), 67(29), 55(31)
VIII	DET	1.08	86(B), 149(10), 130(7), 58(7)
I_a	*N*-TFA-*l*-prolyl-*l*-amphetamine	1.11	166(B), 237(62), 194(40), 91(28)
I_b	*N*-TFA-*l*-prolyl-*d*-amphetamine	1.14	same as for I_a
IX	DMMT	1.15	58(B), 215(5), 160(2)
II_a	*N*-TFA-*l*-prolyl-*l*-methamphetamine	1.23	58(B), 166(86), 251(65), 91(34)
II_b	*N*-TFA-*l*-prolyl-*d*-methamphetamine	1.25	same as for IIa
III_b	*N*-TFA-*l*-prolyl-*d*-norephedrine	1.28	166(B), 238(61), 194(24), 69(20)
III_a	*N*-TFA-*l*-prolyl-*l*-norephedrine	1.30	same as for III_b
IV_b	*N*-TFA-*l*-prolyl-*d*-ephedrine	1.35	58(B), 166(64), 251(19), 69(16)
IV_a	*N*-TFA-*l*-prolyl-*l*-ephedrine	1.37	same as for IV_b
V_a	*N*-TFA-*l*-prolyl-*l*-MDA[c]	1.37	162(B), 166(76), 194(26), 135 (16)
V_b	*N*-TFA-*l*-prolyl-*d*-MDA[c]	1.39	same as for V_a

[a] RRT = relative retention with respect to caffeine.
[b] Numbers in parentheses represent percent abundance with respect to base ions (B).
[c] Assignments of these enantiomers based on assumptions discussed in text.

Figure 2B are listed in Table 1. Although mass spectra previously reported for tryptamine hallucinogens (21), DMMT (22), and *l*-TPC derivatized amphetamine and methamphetamine (12) are helpful, the identifications of these chromatographic peaks are mainly confirmed by comparing their retention times and mass spectra with those obtained from control compounds. Interpretation of fragmentation mechanisms of these compounds is not pursued.

The assignments of the *d*- and *l*-enantiomers of amphetamine, methamphetamine, norephedrine, and ephedrine are certain. These assignments are confirmed by enriching the mixture with a known enantiomer. Because no pure enantiomer of MDA is available, the assignments of MDA enantiomers are based on the assumptions that this enantiomeric pair exhibit correlation between absolute configuration and elution order (23–26), and that the chromatographic behavior of MDA is similar to that of amphetamine. Because structural differences between MDA and amphetamine do not directly involve the asymmetric carbon, it is likely that the diastereomeric derivatives formed will exhibit similar elution characteristics. The reversed elution sequence observed for norephedrine and ephedrine enantiomers is probably related to the second chiral centers in these compounds. Nevertheless, the assignments of MDA enantiomers should still be considered as tentative.

Results presented here clearly demonstrate that mixtures of several compounds related to look-alike drugs and other commonly abused amine drugs and their possible enantiomers can be identified with the use of a chiral derivatizing reagent and a capillary column gas chromatographic–mass spectrometric method. Drug enantiomers are well resolved and acceptable for quantitative analysis with the exception of ephedrine.

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Rapid Colorimetric Assay of Trimethoprim and Sulfamethoxazole in Pharmaceuticals

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A method is described for the direct colorimetric determination of trimethoprim and sulfamethoxazole in pharmaceutical preparations, without prior separation. Estimation of trimethoprim is based on its ion-pair formation with bromophenol blue and subsequent measurement of absorbance of the ion-pair at 418 nm. Estimation of sulfamethoxazole is possible without removal of trimethoprim by solvent extraction.

In view of the extensive application of co-trimoxazole formulations (combination of trimethoprim, an amino pyrimidine antibacterial, with sulfamethoxazole), rapid analysis of trimethoprim and sulfamethoxazole has become necessary. Most official analyses (1, 2) involve an extraction procedure for separating the drugs. In the *British Pharmacopoeia* method of analysis (2), prior separation of sulfamethoxazole has been avoided by specifying amperometric titration of this compound. However, simplicity and rapidity was improved only marginally because the extraction steps could not be totally avoided. A liquid chromatographic method has been developed for such analysis (3), but facilities for carrying out this technique are limited for routine assay, particularly in developing countries. A direct spectrophotometric method (4) does not avoid interference by the light-absorbing impurities that are due to preservatives (methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, etc.) that are generally used in pharmaceutical preparations.

In the present communication, we report a new colorimetric reaction for the assay of trimethoprim which enabled us to avoid any of the extraction steps of the present official methods. Weber et al. reported the formation of an ion-pair when trimethoprim is treated with an acid dye (5). We studied the formation of such an ion-pair of trimethoprim with bromophenol blue. Chloroform could be used successfully for the selective isolation of the reaction product from the reaction mixture for absorbance measurement. It has also been shown that the colorimetric method of the *British Pharmacopoeia* (1) can be used for estimation of sulfamethoxazole directly in the presence of trimethoprim.

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Experimental

Reagents and Apparatus

Citrate-phosphate buffer of pH 3.2 was prepared by mixing 377 mL 0.1M citric acid with 123 mL 0.2M disodium hydrogen phosphate. Solution of bromophenol blue (0.4%, British Drug Houses, UK) was prepared in citrate-phosphate buffer pH 3.2 and stored after filtering. This buffered reagent solution is stable more than 1 week. Sodium nitrite solution (0.1%), 4N HCl, solution of *N*-1-(naphthyl)-ethylenediamine dihydrochloride (0.1%, Koch-Light Laboratories Ltd, UK), and solution of ammonium sulfamate (0.5%) were prepared. Pure samples of trimethoprim (T) and sulfamethoxazole (S) were obtained from Burroughs Wellcome and Co., London, UK. Septran tablets (Burroughs Wellcome and Co., India) and Bactrim pediatric suspension (Roche Products Ltd, India) were purchased from local markets. All reagents were analytical grade.

Absorbance measurements were carried out by using a Bausch and Lomb Spectronic-20 spectrophotometer.

Preparation of Standard

Approximately 20 mg each of (T) and (S) was accurately weighed, dissolved in 2 mL methanol, and diluted to 100 mL with water. This served as the stock standard. Dilutions with water from the stock standard were made separately for (T) and (S) at concentrations of 40 and 3 μg/mL, respectively, to prepare working standards.

Preparation of Samples

Tablets.—Twenty tablets were ground to a fine powder and an accurately weighed portion of the powder equivalent to ca 8 mg (T) and 40 mg (S) was added to 150 mL water in a 200 mL volumetric flask; the mixture was warmed 15 min in a steam bath with intermittent swirling to dissolve the ingredients. The solution was then cooled, 0.8 mL methanol was added, and the so-

lution was diluted to volume with water. This solution served as the final sample dilution for (T). For (S), final dilution was obtained by diluting 1.5 mL of the above solution to 100 mL with water.

Liquid preparations.—An accurately measured volume of the sample equivalent to about 8 mg (T) and 40 mg (S) was treated as described for tablets.

Determination

Assay of (T).—To 2.5 mL standard and sample dilutions in 50 mL glass-stopper cylinders, 1.5 mL water and 2 mL bromophenol blue reagent were added followed by 20.0 mL chloroform. The ion pair formed instantaneously in the aqueous phase, and was extracted in the organic phase by gentle shaking of the mixture for 1 min. The mixture was then allowed to stand for complete separation. The aqueous top layer was discarded and the chloroform layer was carefully transferred to the spectrophotometric cell by using a pipet attached to suitable suction device such as a Griffin pipet filler. Absorbance of the chloroform layer was measured at 418 nm against a reagent blank prepared as described above except that 2.5 mL water was taken instead of standard and sample dilutions.

Assay of (S).—Final standard and sample dilutions (5 mL) were acidified with 1.5 mL 4N HCl, 0.5 mL sodium nitrite solution was added, and the solution was mixed well. Ammonium sulfamate solution (0.5 mL) was added to this mixture after 3 min and the solution was mixed thoroughly. After 2 min, 0.5 mL *N*-1-(naphthyl)-ethylenediamine dihydrochloride reagent was added, and the solution was mixed well and allowed to stand for 10 min for development of color. The absorbance of this solution was then measured at 545 nm against a reagent blank prepared as described above except that 5 mL water was taken instead of standard and sample dilutions.

Results and Discussion

The absorbance maximum of the bromophenol blue-trimethoprim ion-pair extracted by chloroform was 418 nm. At this wavelength, concentration vs absorption plots for the ion-pair containing trimethoprim up to 80 μg/10 mL chloroform followed Beer's Law. Formation of the ion-pair was most favorable when pH of the aqueous phase was 3.2 (Figure 1). The color developed was stable at least 24 h.

Table 1 shows the average recovery of the individual components in the binary mixture

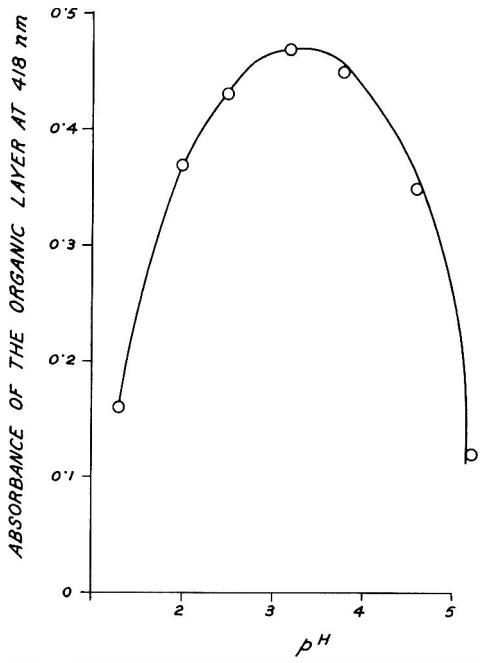

Figure 1. Absorbance (at 418 nm) of the trimethoprim-bromophenol blue ion-pair formed at different pH values (reaction procedure is described in the text).

containing (T) and (S). Trimethoprim and sulfamethoxazole contents in the tablet and oral suspension estimated by the proposed method (Table 2) agree well with the declared amounts of these compounds, and are comparable with results obtained by using extraction-spectrophotometric determination (1). Complete recovery (100%) of sulfamethoxazole from the binary mixture also indicates that the presence of trimethoprim does not interfere with the diazotization and subsequent dye coupling reaction for sulfamethoxazole. In most cases, the present assay method gave marginally higher values than did the conventional extraction method (Table 2). This difference might be due to the loss of materials during extraction. It was further observed that the most common preservatives, such as methyl, ethyl, or propyl *p*-hydroxybenzoate, which are usually present in oral preparations, do not interfere in the analysis for either trimethoprim or sulfamethoxazole by the present method. Thus the procedure is also superior to the direct spectrophotometric method (4) which cannot avoid the interference of such UV-absorbing compounds.

Acknowledgment

We acknowledge our deep sense of gratitude

Table 1. Recovery of trimethoprim and sulfamethoxazole from their binary mixtures

Mixture	Trimethoprim, μg/mL		Sulfamethoxazole, μg/mL	
	Present	Recd	Present	Recd
1	40	40.4	40	40.0
2	30	29.9	50	50.3
3	20	19.9	60	59.8
4	14	14.1	70	70.2
Av., % ± SD		100.2 ± 0.7		100.1 ± 0.4

Table 2. Estimation of trimethoprim and sulfamethoxazole in commercial co-trimoxazole preparations by 2 methods

Product	Compound	Present method[a]		B.P. method[b]	
		Found, mg/tab. or 5 mL suspension	Found, % label claim	Found, mg/tab. or 5 mL suspension	Found, % label claim
Septran tablet[c]	trimethoprim	78.9 ± 0.9	98.6 ± 1.2	78.7 ± 1.3	98.4 ± 1.7
	sulfamethoxazole	399.8 ± 2.2	100.0 ± 0.5	396.2 ± 3.2	99.0 ± 0.8
Bactrim pediatric suspension[d]	trimethoprim	39.3 ± 0.6	98.2 ± 1.5	39.6 ± 0.5	99.1 ± 1.4
	sulfamethoxazole	199.8 ± 2.5	99.9 ± 1.2	198.0 ± 2.1	99.0 ± 1.0

[a] Results are mean of 10 independent determinations ± SD.
[b] Results are mean of 5 independent determinations ± SD.
[c] Label claim = 80 mg trimethoprim and 400 mg sulfamethoxazole/tablet.
[d] Label claim = 40 mg trimethoprim and 200 mg sulfamethoxazole/5 mL.

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First-Derivative Spectroscopic Determination of Acetaminophen and Sodium Salicylate in Tablets

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A first-derivative spectroscopic method for the simultaneous determination of acetaminophen and sodium salicylate in tablets was developed. Solutions of this drug combination in acidic ethanol were analyzed using their respective spectral responses at 258.5 and 317.0 nm. The method, which can be used for tablet composite assay and content uniformity analysis, is linear for acetaminophen concentrations ranging from 0.0 to 21.6 μg/mL, and for sodium salicylate concentrations ranging from 0.0 to 36.0 μg/mL. Relative standard deviations for the assay of both drugs in commercial tablets were <2%, and recoveries of acetaminophen and sodium salicylate from spiked samples were 99.7 and 100.1%, respectively. The results obtained by first-derivative spectroscopy were in agreement with the results of a liquid chromatographic procedure for acetaminophen and a fluorometric method for sodium salicylate. The technique used for the selection of wavelengths for analysis is also described.

The use of a mixture of acetaminophen and sodium salicylate as an analgesic is well established in humans. In the United States, tablets of this mixture are sold over-the-counter to be used to treat rheumatoid arthritis, osteoarthritis, fibrositis, bursitis, and related conditions (1). While compendial methods are available for the separate quantitation of acetaminophen and sodium salicylate in various forms (2, 3), no official method could be found for their determination in combination.

For this reason, a simple, quick, and reliable method for the assay and content uniformity testing of this drug combination in dosage forms is needed. Published data on the spectroscopic characteristics of these 2 substances (4) indicate that their corresponding ultraviolet (UV) absorption maxima differ by approximately 50 nm, a difference which is both pH- and solvent-dependent. Although the individual UV absorption spectra of these 2 compounds overlap in the region of 210–320 nm (Figure 1), the aforementioned spectral characteristics suggested that derivative spectroscopy (5–7) could be used to simultaneously determine both drugs without prior separation from each other.

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Derivative UV absorption spectroscopy has been used to quantitate several compounds of biological interest by reducing many of the spectral interferences encountered in conventional spectrophotometric methods (8–12). Pharmaceutical applications of derivative spectroscopy are few (6) and, for the most part, have been limited to enhancing the spectral features of a drug to facilitate its identification (13, 14) or quantitation (15). Several papers (6, 16–18) have reported the successful application of this technique to the determination of single-component pharmaceutical dosage forms containing excipients that interfere with their spectrophotometric analysis. Only a few reports (6, 19, 20) have dealt with the application of derivative spectroscopy to the simultaneous determination of more than one active ingredient in multicomponent pharmaceutical dosage forms.

This paper presents a first-derivative spectroscopic method, based on the "zero-crossing technique" (5, 12), which is capable of simultaneously determining acetaminophen and sodium salicylate in tablets.

Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Cary 219 UV/Vis with derivative capability (Varian Instrument Div., Palo Alto, CA 94303), or equivalent. Operating parameters: function dA; dA range 10 abs/min; scan rate 2 nm/s; chart display 5 nm/cm; scanning range 360–240 nm; cell path 10 mm; period 1.0 s; lamp source UV. With these parameters, 1.0 abs/min = 5% full-scale deflection of recorder.

(b) *4N Ethanolic HCl.*—To ca 100 mL USP grade ethanol in 200 mL volumetric flask, add 6.8 mL HCl. Dilute to volume with ethanol and mix.

(c) *Acetaminophen standard.*—Chemically pure laboratory working standard meeting compendial requirements (3) and dried for 18 h over silica gel before use.

(d) *Sodium salicylate standard.*—Chemically pure laboratory working standard meeting compendial requirements (3) and dried for 4 h at 105°C before use.

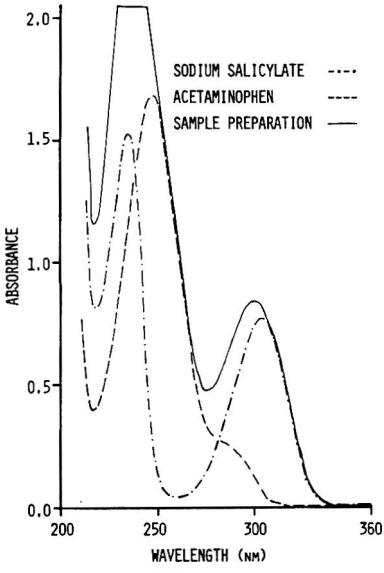

Figure 1. UV absorption spectra of acetaminophen standard solution (18 μg/mL), sodium salicylate standard solution (30 μg/mL), and sample solution (with nominal concentration of 18 μg acetaminophen/mL and 30 μg sodium salicylate/mL). Reference: solvent blank.

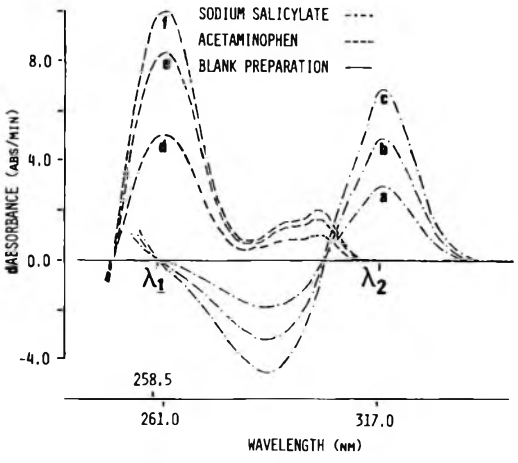

Figure 2. Graphical determination of suitable analytical wavelengths for simultaneous quantitation of acetaminophen and sodium salicylate mixtures. Sodium salicylate calibration solutions: (a) 18.0 μg/mL, (b) 30.0 μg/mL, and (c) 42.0 μg/mL. Acetaminophen calibration solutions: (d) 10.8 μg/mL, (e) 18.0 μg/mL, and (f) 21.6 μg/mL.

Selection of Wavelengths

Preparation of calibration solutions.—Prepare at least 3 solutions of each drug by weighing quantities of each standard into separate 100 mL volumetric flasks. Weights of each standard should be chosen so that concentration range of these solutions spans expected concentration range of sample preparation (i.e., 1.8, 3.0, and 4.2 mg sodium salicylate represent 60, 100, and 140% of declared amount of drug in sample preparation of tablets labeled to contain 300 mg sodium salicylate, and diluted by a factor of 10 000). To each flask add ca 40 mL ethanol and dissolve drug with sonication. Add 4.5 mL water and 5 mL 4N ethanolic HCl, mix, and dilute to volume with ethanol. Prepare solvent blank in same manner or use 0.0 μg/mL mixed standard solution. Since these solutions will be used only to determine isoabsorptive points, standards need not be weighed accurately.

Spectrophotometry.—Superimpose first-derivative absorption spectra of various concentrations of acetaminophen and sodium salicylate calibration solutions and of solvent blank on same chart paper (Figure 2). Examine spectral curves and locate wavelength (ca 258.5 nm) at which sodium salicylate curves show an isoabsorptive point, that is, where dAbsorbance (change in absorbance accompanying incremental change in wavelength) = 0. This wavelength, labeled "λ_1", is near dAbsorption maximum of acetaminophen (ca 261.0 nm) and can therefore be used for its quantitation because sodium salicylate does not interfere. Examine spectral curves once more and locate dAbsorption maximum for sodium salicylate (ca 317.0 nm) and label this wavelength "λ_2." Sodium salicylate can be accurately measured at this wavelength because acetaminophen does not interfere; that is, its dAbsorbance = 0 at this wavelength.

Preparation of Standards

Mixed standard stock solution.—Accurately weigh suitable quantities of acetaminophen and sodium salicylate standards into volumetric flask. Dissolve and dilute to volume with ethanol to obtain solution containing ca 360 μg acetaminophen/mL and 600 μg sodium salicylate/mL.

Mixed standard working solutions.—Into separate 100 mL volumetric flasks containing 4.5 mL water, pipet 0.0, 4.0, 5.0, and 6.0 mL aliquots of mixed standard stock solution to give concentrations of 0.0, 14.4, 18.0, and 21.6 μg acetaminophen/mL and 0.0, 24.0, 30.0, and 36.0 μg sodium salicylate/mL, respectively. To each flask add ca 40 mL ethanol, followed by 5 mL 4N ethanolic HCl. Dilute to volume with ethanol and mix.

Preparation of Samples

Composite assay.—Weigh and finely powder

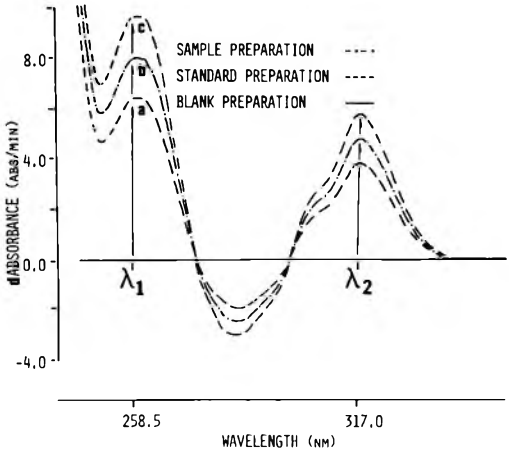

Figure 3. Measurement of "blank corrected" dAbsorbance for acetaminophen and sodium salicylate in sample and standard solutions. Mixed standard concentrations: (a) 24.0 μg sodium salicylate/mL and 14.4 μg acetaminophen/mL; (b) 30.0 μg sodium salicylate/mL and 18.0 μg acetaminophen/mL; (c) 36.0 μg sodium salicylate/mL and 21.6 μg acetaminophen/mL. (In this diagram, the sample solution overlaps mixed standard curve "b".)

≥20 tablets. Calculate average tablet weight. Accurately weigh portion of powdered sample, equivalent to ca 1 average tablet weight, and place in 500 mL volumetric flask. Add 50 mL ethanol and stir 15 min at ca 60°C. Carefully add 50 mL boiling water, and continue stirring and heating for additional 15 min. Let cool to room temperature and dilute to volume with water. Filter solution through fast paper (Whatman No. 2), discarding first 25 mL filtrate. Transfer 5.0 mL clear filtrate to 100 mL volumetric flask containing ca 40 mL ethanol. Add 5 mL 4N ethanolic HCl, dilute to volume with ethanol, and mix.

Individual tablet assay.—Select suitable number of tablets for analysis (3). Fragment each tablet and quantitatively transfer to separate 500 mL volumetric flasks. Proceed according to directions given for *Composite Assay*, starting with "Add 50 mL ethanol . . . ".

Recovery study.—Accurately weigh portion of powdered composite sample equivalent to ca 60% average tablet weight and transfer to 500 mL volumetric flask. Add accurately weighed amounts of acetaminophen and sodium salicylate standards, equivalent to ca 40% declared amounts of these drugs per tablet. Treat this mixture in same manner described for *Composite Assay*, starting with "Add 50 mL ethanol . . . ".

Determination

Using identical instrumental settings, scan first-derivative absorption spectra of sample solution and mixed standard working solutions vs solvent blank. To quantitate acetaminophen, prepare linear regression plot of acetaminophen standard concentrations vs their corrected dAbsorbances at λ_1 (Figure 3). Calculate amount of acetaminophen per average tablet weight from acetaminophen regression plot. To quantitate sodium salicylate, prepare linear regression plot of sodium salicylate standard concentrations vs their corrected dAbsorbances at λ_2 (Figure 3). Calculate amount of sodium salicylate per average tablet weight from sodium salicylate regression plot.

Results and Discussion

The first-derivative spectroscopic method described here permits the rapid, precise, and accurate simultaneous determination of acetaminophen and sodium salicylate in commercial tablets. A solvent system that would place a dAbsorption maximum of one component at or very near the zero-crossing wavelength(s) of the other component(s) was needed. Experimentation with a variety of solvent systems showed that dilute solutions of mineral acids in either ethanol or methanol gave the optimum solvent conditions for the simultaneous determination of the 2 drugs in pharmaceutical combinations.

During the preparation of the samples for analysis, aliquots of sample and standard stock solutions were diluted with ethanol before the addition of ethanolic HCl. In this manner, the possibility of hydrolysis of the acetaminophen by the direct addition of strong acids was precluded. Dilute solutions of acetaminophen in 0.2N ethanolic HCl degraded upon standing for >24 h, as shown by the progressive appearance of distortions in its first-derivative UV spectrum. Dilute solutions of sodium salicylate in the same medium, however, were stable for at least 1 week.

Least-squares regression analysis of dAbsorbance vs concentration for both acetaminophen (Table 1) and sodium salicylate (Table 2) gave linear plots over the concentration range 0–120% of the declared amount of each drug in commercial tablets. Moreover, these plots passed through the origin, indicating the absence of interfering absorbing species.

The precision of the proposed method was evaluated by assaying the acetaminophen and sodium salicylate content of 4 accurately

Table 1. Correlation between acetaminophen concentration and dAbsorbance measured at 258.5 nm (concentration factor = 1.000)

Nominal concn, μg/mL	dAbs found[a]	dAbs calcd by least-squares	Deviation, %
0.0	0.0	−0.03	—
14.4	32.7	32.70	0.0
18.0	40.7	40.88	0.4
21.6	49.2	49.06	0.3
	Correlation coefficient = 0.99998		

[a] Measured as percent of full scale and corrected for absorption of blank (see Figure 3).

Table 2. Correlation between sodium salicylate concentration and dAbsorbance measured at 317.0 nm (concentration factor = 1.000)

Nominal concn, μg/mL	dAbs found[a]	dAbs calcd by least-squares	Deviation, %
0.0	0.0	−0.03	—
24.0	19.4	19.50	0.5
30.0	24.4	24.38	0.1
36.0	29.3	29.26	0.1
	Correlation coefficient = 0.99999		

[a] Measured as percent of full scale and corrected for absorption of blank (see Figure 3).

weighed portions of powdered tablet composite sample, each equivalent to approximately 1 average tablet weight. The results of this study are presented in Table 3. The relative standard deviations for both drugs were <2%. Recoveries by the proposed method of known amounts of acetaminophen (73.0 mg) and sodium salicylate (120.5 mg) added to a powdered tablet composite sample were 99.7 and 100.1%, respectively.

Ten commercial tablets labeled to contain 180 mg acetaminophen and 300 mg sodium salicylate/tablet were analyzed by the proposed method. The average content of acetaminophen and sodium salicylate in these tablets was 175.7 and 286.8 mg, respectively (Table 4). These values agree with the average assay results for the powdered tablet composite sample.

The accuracy of this first-derivative spectroscopic method was also tested by comparing its results with those obtained by 2 other analytical procedures (Table 5). Results for sodium salicylate were in agreement with those obtained using a modification of the fluorometric procedure of Weissbach et al. (21). The values for acetaminophen were in accord with those obtained using the liquid chromatographic (LC) method described by D. J. Krieger (Food and

Table 3. Assay results for tablet composite sample

	Acetaminophen, 180 mg/tab.		Sodium salicylate, 300 mg/tab.	
Detn no.	Found per av. tablet, mg	Found, % of decld	Found per av. tablet, mg	Found, % of decld
1	179.2	99.6	297.7	99.2
2	172.4	95.8	289.6	96.5
3	174.8	97.1	291.4	97.1
4	172.3	95.7	285.7	95.2
Av.	174.7	97.0	291.1	97.0
Rel. std dev., %	1.8		1.7	

Table 4. Assay results for individual tablets

	Acetaminophen, 180 mg/tab.		Sodium salicylate, 300 mg/tab.	
Tablet no.	Found per tab., mg	Found, % of decld	Found per tab., mg	Found, % of decld
1	169.8	94.3	281.0	93.7
2	180.5	100.3	290.7	96.9
3	172.0	95.6	285.8	95.3
4	177.1	98.4	293.2	97.7
5	171.5	95.3	281.0	93.7
6	179.2	99.6	287.1	95.7
7	175.0	97.2	285.8	95.3
8	172.4	95.8	282.2	94.1
9	183.5	101.9	295.6	98.5
10	175.8	97.7	285.8	95.3
Av.	175.7	97.6	286.8	95.6
Rel. std dev., %	2.5		1.7	

Table 5. Comparison of assay results for acetaminophen and sodium salicylate in tablets by 3 methods

Method	No. of assays	Acetaminophen, 180 mg/tab.		Sodium salicylate, 300 mg/tab.	
		Av. found, mg/av. tab.	Av. found, % of decld	Av. found, mg/av. tab.	Av. found, % of decld
Derivative spectroscopy	4	174.7	97.0	291.1	97.0
LC	2	180.0	100.0	—	—
Fluorescence	2	—	—	294.0	98.0

Drug Administration, 1982, private communication).

By using a modified version of the test described by Doyle and Fazzari (22), the proposed method was shown to be free of interference from other tablet components. The first-derivative UV absorption spectrum of the sample solution intersected the baseline (dAbsorbance = 0) at all points predicted by the mixed standard working solutions (Figure 3). If the sample solution had not demonstrated this property, this would have indicated that sample and standard solutions differed in some respect other than concentration (which does not affect these points). In this event a preliminary separation of the drug(s) from excipients, or from each other, would have been required before UV spectroscopic determination (22).

The ability of derivative spectroscopy to selectively enhance the sharper spectral features of a chromophore (7) has been used advantageously in pharmaceutical analysis to assay drugs with poorly defined absorption maxima (15) or when excipients or other active ingredients in the dosage form interfered with conventional spectrophotometric determinations (6, 16–18). This paper demonstrates the potential of derivative spectroscopy as an analytical technique and its usefulness to rapidly and simultaneously quantitate active ingredients in multicomponent pharmaceuticals.

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Spectrophotometric Determination of Hydralazine Hydrochloride Tablets Using Ninhydrin

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A method is described for the assay of hydralazine HCl in tablets, based on the colored product formed by the reaction between hydralazine and ninhydrin. The reaction is conducted at room temperature in a pH 3 buffer solution and the colored product is measured spectrophotometrically at the absorption maximum at 442 nm. Under the stipulated conditions, this reaction is highly specific for hydralazine and is not affected by other drugs which may be used in combination with hydralazine. Results of infrared and mass spectrometric studies suggested that the colored product is a hydrazone. Ultraviolet spectrophotometry also showed that dilute solutions of hydralazine degrade rapidly in the presence of alcohols.

Hydralazine is an antihypertensive drug. It can be assayed by iodate titration (1) or by spectrophotometric methods (2, 3). The determination of hydralazine in body fluids (2) and in related drugs (4), based on the color development with ninhydrin at 70°C and 80°C, has been reported. However, at these elevated temperatures, a number of drugs such as naphazoline, phenylpropanolamine, ergotamine, ergometrine, nitrazepam, flurazepam, and diazepam also react with ninhydrin to give colored complexes, so the method lacks specificity.

This paper describes a rapid and accurate spectrophotometric determination of hydralazine hydrochloride at room temperature and presents mass spectrometric and infrared data on the colored complex formed in the reaction with ninhydrin.

METHOD

Apparatus and Reagents

(a) *Ultraviolet-visible spectrophotometer.*—Perkin Elmer Coleman 570.

(b) *Mass spectrometer.*—Hewlett-Packard HP 5895B, with direct insertion probe at 200°C.

(c) *Infrared spectrophotometer.*—Perkin Elmer 399B.

(d) *Ninhydrin solution.*—10% ninhydrin (Merck, Darmstadt, GFR) in methanol (AR grade). Store in refrigerator.

(e) *Buffer solution.*—Dissolve 40.846 g potassium hydrogen phthalate in water and dilute to 1 L to give 0.2M solution. To 50 mL, add 20.32 mL 0.2M HCl and dilute to 200 mL with water to give a buffer solution of pH 3.

(f) *Hydralazine standard solution.*—0.002% hydralazine HCl (USP Reference Standard) in pH 3 buffer solution, freshly prepared.

Procedure

Powder known number of tablets and accurately weigh quantity of powder containing ca 0.15 g hydralazine HCl. Transfer to 100 mL standard flask and dilute to mark with buffer solution. Shake 15 min, filter, and pipet 10 mL filtrate into 100 mL standard flask; dilute to mark with buffer solution. Pipet 4 mL of this solution into 50 mL standard flask, add 6 mL buffer solution and 2 mL of ninhydrin solution, and swirl. Concurrently, prepare reagent blank of 10 mL buffer and 2 mL ninhydrin solution. Let solutions stand in dark at room temperature (27°C) for 1 h, then dilute to mark with water, and measure absorbance at 442 nm against reagent blank. Establish concentration by reference to calibration graph prepared by following same procedure except omit filtration step. Also, add buffer solution to reaction mixture in an amount so that total volume of reaction mixture, before addition of 2 mL ninhydrin solution, is 10 mL.

Results and Discussion

Nature of Complex

When an ethanolic solution of ninhydrin was added to a strong aqueous solution of hydralazine HCl at room temperature, a bright red stable complex was formed and precipitated from solution. The electron impact mass spectrum of the complex showed major fragments at m/z 302 (molecular ion), 273, 245, 158, 145, 130, 117, 103, 89, and 76. The infrared spectrum had strong and sharp absorption bands at 1670, 1580, 1480, 1430, 1310, 1330, 1130, 980, and 740 cm^{-1}. These data suggested that the complex is a simple hydrazone addition reaction product as shown in Figure 1.

However, when the reaction was conducted with low concentrations of hydralazine HCl (up to 10 mL of 0.004% aqueous solution) and 2 mL

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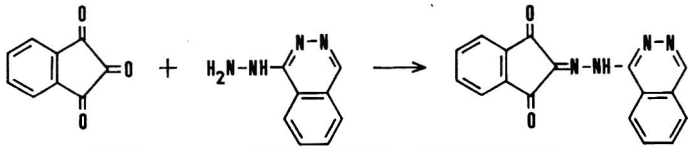

Figure 1. Reaction of ninhydrin with hydralazine.

10% ninhydrin solution in methanol, the complex remained in solution and exhibited an absorption maximum at 442 nm.

Stability of Hydralazine Hydrochloride

The stability of hydralazine hydrochloride in both aqueous and organic solvents was studied through changes in its ultraviolet spectrum. It was observed that at a strength of 0.001% in 50% (v/v) methanol (i.e., dilute methanol 1 + 1), there were significant changes with time for the major peak at 240 nm and the shoulder at 280 nm. After 3 h of standing at room temperature, there was about 30% increase in absorbance at 240 nm but that at 280 nm remained essentially the same. After 6 h, the increase at 240 nm was 50%, while at 280 nm there was an increase of about 70% to form a new peak. At the end of 24 h, the 240 nm peak was 3 times as high but the absorbance at 280 nm was below its original value. For a 1% solution of the compound in 50% methanol, there were no significant spectral changes on standing. However, once diluted, the same pattern of changes occurred. These observations are significant when testing the uniformity of content of hydralazine HCl tablets by the U.S. Pharmacopeia (USP XX) method (1) which uses 50% (v/v) methanol to dissolve the tablets. Hence, weak solutions of hydralazine in 50% methanol should not be left to stand for more than $\frac{1}{2}$ h.

Solutions of 0.001% w/v of hydralazine HCl in ethanol and other alcohols also showed spectral changes on standing. However, no spectral or absorbance changes were observed for 0.001% w/v solutions in water up to 48 h. In pH 3 buffer solution, hydralazine HCl was stable for about 24 h.

Effect of pH on Color

There was significant loss of color when the reaction was conducted at pH between 5.5 and 7.0. At pH 8 (borate buffer), although color intensity was the highest, reproducibility and linearity were not at an optimum. At pH 3, although absorbance values were about 30% lower, linearity and reproducibility were excellent. The color, after 1 h development, showed no change in absorbance for up to 4 h.

Effect of Reagent Concentration

The results in Figure 2 show that the absorbance increases with increasing amounts of 10% ninhydrin reagent. However, at 2 mL, it reaches a plateau. Hence, 2 mL of ninhydrin solution was used in all determinations.

Specificity of Reaction

Ninety-two common drugs including naphazoline, phenylpropanolamine, ergotamine, ergometrine, nitrazepam, flurazepam, diazepam, medazepam, reserpine, guanethidine, hydrochlorothiazide, chlorothiazide, methyldopa, and triamterene were tested under the conditions of the proposed method and, other than medazepam, none of the compounds exhibited any color. Medazepam gives a yellow color when dissolved in the buffer solution and therefore can be distinguished from hydralazine. The reaction is, therefore, highly specific under the conditions of the proposed method. Diuretics and other anti-hypertensives likely to be found in combination with hydralazine, such as chlorothiazide, hydrochlorothiazide, triamterene, guanethidine, methyldopa, and reserpine, do not interfere even when present at a ratio of 50:1 against hydralazine hydrochloride.

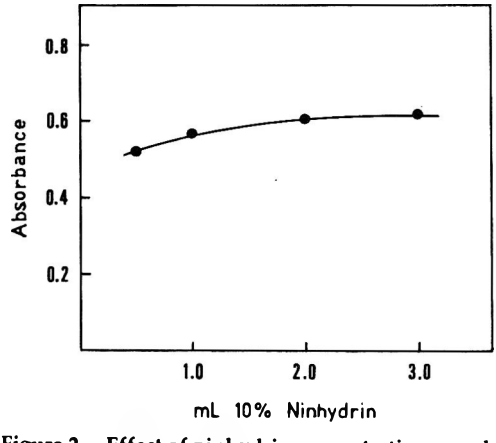

Figure 2. Effect of ninhydrin concentration on color development (absorbance at 442 nm) of 208 μg hydralazine HCl.

Table 1. Determination of hydralazine HCl in tablets

Brand/Source	Content	Stated amt per tab., mg	Hydralazine HCl found, mg/tab.[a]	
			Proposed	USP XX
Hyperex 50	hydralazine HCl	50	51.7	51.7
			52.1	52.2
Hyperex 25	hydralazine HCl	25	25.5	24.8
			25.7	25.1
Pharm. Dept Singapore	hydralazine HCl	10	9.9	9.7
			9.9	9.8
SER-AP-ES	reserpine	0.1		
	hydralazine HCl	25	23.7	23.6
			23.8	23.8
	hydrochlorothiazide	15		
Apresoline 50	hydralazine HCl	50	49.8	49.1
			49.2	49.7

[a] Each value represents one determination.

Calibration Graph and Sensitivity

Calibration graphs were constructed in the usual way and Beer's law was obeyed at 442 nm for 0–280 μg of hydralazine HCl in 50 mL solution. The correlation coefficient was 0.9997 and the calibration graph can be represented by the equation $A = 2.878x + 0.002$, where A is the absorbance at 442 nm and x is mg hydralazine hydrochloride in 50 mL solution. The day-to-day variation of the calibration graph was less than 1.0%. Consequently, the value for the absorptivity with respect to hydralazine hydrochloride was calculated to be 143.9 ± 1.4.

Recovery Studies

The method was applied to the determination of hydralazine in both hydralazine tablets and compound tablets containing hydralazine. The results were compared with those obtained by the USP XX methods (1). As shown in Table 1, the results are in good agreement. Statistical analysis based on Students' t-test for the 2 sets of data indicated that there was no significant difference between the methods.

Comparison with Other Methods

The proposed method is simple as there is no need for solvent extraction or extensive shaking. While for some 10 mg hydralazine tablets, the use of the USP XX titration method can present difficulty in the determination of the colored end point in chloroform, the proposed method performed satisfactorily. Furthermore, the proposed method can also be successfully applied to compound tablets and other samples of low hydralazine contents. An important advantage of this assay over the other ninhydrin spectrophotometric assays (2, 4) is that no temperature control at elevated temperature is required.

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MYCOTOXINS

Rapid Liquid Chromatographic Determination of Aflatoxins in Heavily Contaminated Corn

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A procedure is described for rapid, quantitative determination of aflatoxins B_1, B_2, G_1, and G_2 in heavily contaminated corn (>500 μg B_1/kg) from field, greenhouse, and growth chamber experiments employing artificial inoculation of corn with *Aspergillus flavus*. Whole kernel corn is ground to pass a 1 mm screen and mixed before extraction of a water-wetted (25 mL) 50 g subsample with 250 mL chloroform. The filtered extract is diluted 1:1 with hexane and applied to a hexane-wetted (10 mL) disposable silica cartridge. Interferences are removed with chloroform-hexane (1 + 3), and aflatoxins are quantitatively eluted with hexane-acetone (1 + 1). Aflatoxins B_1 and G_1 are converted to the more intensely fluorescent hemiacetals, B_{2a} and G_{2a}, by treatment with trifluoroacetic acid-water. Derivatized aflatoxins are separated by reverse phase liquid chromatography (LC) and quantitated fluorometrically. Compared with AOAC method I (CB) for corn, using samples containing approximately 50 and 10 000 μg B_1/kg, agreement between methods was good at the lower level while the rapid method yielded a considerably larger mean at the higher level. A precision study of 30 replicate samples produced a coefficient of variation of 8.46% at a mean value of 1066 μg B_1/kg. The cartridge method was developed for LC analysis of samples that contain >500 μg aflatoxin B_1/kg corn, but it may be used to quantitate as little as 10 μg B_1/kg with no modification.

Aflatoxin contamination of field corn (*Zea mays* L.) at harvest and in storage is a serious problem in the southeastern United States (1). Losses to grain-, feed-, and animal-producing industries in North Carolina attributable to aflatoxin probably exceed $200 million annually (T. E. Nichols, Jr, North Carolina State University, Raleigh, NC, 1982). The NCSU School of Agriculture and Life Sciences as well as other state, federal, and private agencies in North Carolina are responding to this mycotoxin problem with extensive research efforts. Field and laboratory experiments, grain and feed surveys, and other monitoring programs generate several thousand samples each year in which aflatoxins must be quantitatively determined.

Probably the most reliable and generally used method for aflatoxin analysis in corn is AOAC official method I (CB), **26.026-26.031** (2). The CB method is often used as a standard of excellence against which new methods are judged (3-7). Unfortunately, the CB method requires nearly 1 L of expensive solvents per determination for extraction, column preparation, and column chromatography. When heavily contaminated materials are encountered, both repeated dilution and repeated thin layer chromatography (TLC) are generally required to ensure that determinations are made within the linear range of the densitometric quantitation system used, which usually does not exceed 10 ng B_1 per spot. In our laboratory, 2 analysts are required to process about 25 samples per day. The economical, rapid, large scale analysis of heavily contaminated materials by the AOAC method is consequently difficult.

Research samples at NCSU are drawn from field, greenhouse, and growth chamber experiments employing artificial inoculation of corn with *Aspergillus flavus* Link ex. Fries. Because aflatoxin production in living tissues may exhibit wide variation, statistical considerations require experiments using large numbers of plants, replications, and locations. The concentration of aflatoxin B_1 in some of these special samples may exceed 100 mg/kg. While the CB method is applicable to determination of as little as 1 to 10 μg B_1/kg, the upper limit of applicability has not been made clear. The collaborative study by Shotwell and Stubblefield (8), which formed the basis for the decision to use the CB method as the official method for aflatoxin in corn, used levels of aflatoxin contamination below 0.5 mg B_1/kg. The large number of samples and high levels of

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aflatoxin encountered by this laboratory made the development of a reliable, more rapid, and more economical method for the determination of aflatoxins in corn essential.

A purification method using silica cartridge chromatography which would yield an extract suitable for automated LC analysis was sought. Cartridge methods typically require less glassware, less solvent, and less time than does conventional column chromatography. Several methods that specify use of disposable silica cartridges have been published (7, 9–11); however, there were no indications that the methods described would be applicable to levels of aflatoxin contamination as high as those encountered in experiments here. The need for data on the ability of small silica cartridges to adsorb microgram quantities of aflatoxins led to development of this method.

METHOD

Apparatus

(a) *Sample concentrator.*—Model 112 24-position N-EVAP analytical evaporator (Organomation Associates, Inc., Northborough, MA 01532).

(b) *Liquid chromatograph.*—Two M6000A pumps, Model 710B WISP automatic injector, Model 720 system controller, Model 730 data module, with Intelink (Millipore-Waters, Milford, MA 01757).

(c) *Liquid chromatographic column.*—10 cm × 8 mm Radial-Pak A 10 μm C_{18} or 10 cm × 8 mm Radial-Pak A 5 μm C_{18} cartridge and C_{18} Guard-Pak guard column insert used with Model RCM-100 radial compression module (Millipore-Waters).

(d) *Fluorescence detector.*—Model FS950 Fluoromat with excitation at 360 nm using FSA403 bandpass filter and FSA111 lamp and emission above 418 nm using FSA426 longpass filter, with FSA986A automatic overload reset control (Kratos-Schoeffel Instruments, Westwood, NJ 07675).

(e) *Shaker.*—Model 75 12-position wrist-action shaker (Burrell Corp., Pittsburgh, PA 15219).

(f) *Syringe.*—For cartridge chromatography: 50 mL Multifit reusable glass syringe with Luer-lok tip (Fisher Scientific Co.).

(g) *Syringe.*—For preparation of samples for LC analysis: 5 mL disposable polypropylene syringe with Luer-lok tip (Becton, Dickinson and Co., Rutherford, NJ 07070).

(h) *Vials.*—Fisherbrand 20 mL scintillation vial with cork-back foil-lined cap (Fisher Scientific Co.).

(i) *Filter.*—For preparation of samples for LC analysis: 0.45 μm pore size Acrodisc-CR chemical resistant disposable filter assembly (Gelman Filtration Products, Ann Arbor, MI 48106).

(j) *Thin layer chromatography plates.*—E. Merck No. 5763, 20 × 20 cm glass analytical plates precoated with 0.25 mm layer of silica gel 60, without fluorescent indicator (American Scientific Products, Charlotte, NC 28210).

(k) *Spectrophotometer.*—Perkin-Elmer (Coleman 111) UV-Vis spectrophotometer (Hitachi, Ltd., Tokyo, Japan).

(l) *Spotmeter densitometer.*—Instrument developed and described by Dickens et al. (12).

Reagents

(a) *Aflatoxins.*—Aflatoxins B_1, B_2, G_1, and G_2 purchased in 10 mg quantities (Calbiochem-Behring Corp., San Diego, CA 92112) and quantitated spectrophotometrically as in **26.004-26.012** (2). *Warning:* Aflatoxins are potential carcinogens in humans. Observe precautions outlined in Chapter 26 (2).

(b) *Organic solvents.*—ACS grade benzene, ethyl acetate, chloroform, methanol, ethyl ether, hexane, and acetone (Fisher Scientific Co.). HPLC grade acetic acid (J. T. Baker Chemical Co., Phillipsburg, NJ 08865). HPLC grade acetonitrile, methanol, and 2-propanol (Fisher Scientific Co.). *Warning:* Use organic solvents only with proper ventilation. Chloroform is suspected to be carcinogenic in humans. Follow proper safety procedures when using chloroform.

(c) *Water.*—Deionized and HPLC grade provided by Model 1000 Hydro Ultrapure water system (Hydro Service and Supplies, Inc., Research Triangle Park, NC 27709).

(d) *Adsorbent.*—Sep-Pak silica cartridge (Millipore-Waters).

(e) *Trifluoroacetic acid (TFA).*—97% minimum (Eastman Kodak Co., Rochester, NY 14650). *Danger:* Causes burns. Harmful if inhaled. Do not get in eyes, on skin, or on clothing. Use only with adequate ventilation.

(f) *Diatomaceous earth.*—Celite 545 (Fisher Scientific Co.).

(g) *Solutions.*—(*1*) Rinse solution for cartridge chromatography: hexane–chloroform (3 + 1 v/v). (*2*) Elution solvent for cartridge chromatography: hexane–acetone (1 + 1 v/v). (*3*) Mobile phase for LC separation of aflatoxins on 10 μm C_{18} column (2.0 mL/min): 70 mL acetic acid, 70 mL acetonitrile, 70 mL 2-propanol plus water to make 1 L solution. (*4*) Mobile phase for

LC separation of aflatoxins on 5 μm C_{18} column (1.5 mL/min): 20 mL acetic acid, 100 mL acetonitrile, 100 mL 2-propanol plus water to make 1 L solution. Mobile phases should be mixed well and filtered (0.5 μm pore glass fiber filter) before use. (5) Developer for TLC of derivatized aflatoxins: ethyl acetate–ethyl ether–methanol–water (95 + 30 + 2 + 1 v/v/v/v). (6) Derivatization reagent: trifluoroacetic acid–water (9 + 1 v/v).

Extraction

Add 25 mL deionized water, ca 25 g diatomaceous earth, and 250 mL chloroform to 50 g corn sample which has been ground to pass 1 mm screen. Stopper and shake 30 min on wrist-action shaker. Gravity-filter through fluted 33 cm diameter coarse-grade paper.

Cartridge Chromatography

Measure 25 mL filtered extract into 50 mL graduated cylinder. Bring total volume to 50 mL with hexane. Pour contents of cylinder into 125 mL Erlenmeyer flask and mix well by swirling flask vigorously. Attach silica Sep-Pak cartridge to 50 mL syringe. Rinse cartridge with 10 mL hexane. Pass 15 mL aliquot of filtrate–hexane mixture through silica cartridge. Regulate rate of application by depressing syringe plunger until distinct droplets emerging from the cartridge just begin to form continuous stream. Rinse cartridge twice with 15 mL portions of hexane–chloroform (3 + 1). Discard rinses. Elute aflatoxins with single 15 mL portion of hexane–acetone (1 + 1); collect this final cartridge effluent in 20 mL scintillation vial. Evaporate extract to dryness in concentrator (35°C water bath; use gentle flux of nitrogen or dry, filtered air).

Derivatization

Evaporate extract to dryness in concentrator. Add 1.5 mL trifluoroacetic acid–water (9 + 1). Cap and shake to redissolve entire extract. Incubate 15 min at room temperature. Evaporate to dryness in concentrator (ca 35°C water bath; gentle flow of nitrogen or dry, filtered air).

Dissolve 10 mg standard in 50 mL acetonitrile and transfer to 50 mL volumetric flask to yield approximate concentration of 0.2 mg/mL in stock solution. To determine concentration spectrophotometrically, evaporate 0.5 mL stock solution to dryness and redissolve in 20 mL methanol. Proceed as in **26.004–26.012** (2). Use stock solutions to prepare standards containing 200 μg B_1, 100 μg B_2, 100 μg G_1, and 100 μg G_2. Proceed as in derivatization of extracts. Redissolve in acetonitrile to give concentration of 6 ng/μL B_1 (as B_{2a}), 3 ng/μL B_2, 3 ng/μL G_1 (as G_{2a}), and 3 ng/μL G_2.

Thin Layer Chromatography

For underivatized extracts, redissolve extract in 1 mL benzene–acetonitrile (98 + 2 v/v). Proceed as in **26.031** (2).

For derivatized extracts, proceed as indicated for underivatized extracts except develop in ethyl acetate–ethyl ether–methanol–water (95 + 30 + 2 + 1).

LC Determination of Aflatoxins in Derivatized Extract

Redissolve sample in 3 mL acetonitrile. Attach disposable filter unit to 5 mL syringe. Pass sample through filter directly into LC vial; filtration loss is <300 μL. Set fluorescence detector to give about 90% full scale deflection (about 14 cm peak height on data module chart paper) when 5 μL derivatized standards are injected (equivalent to 12 mg B_1/kg, 6 mg G_1/kg, 6 mg B_2/kg, 6 mg G_2/kg or 30 ng B_1, 15 ng G_1, 15 ng G_2, 15 ng B_2). Representative parameter values are: sensitivity = 6.0; range = 0.5; background suppression = Lo; time constant = 5. Actual settings will vary with lamp used.

Set data module for manual integration with peak width 30 s, noise rejection 100, area rejection 100, chart speed 0.25 cm/min. Perform single point calibration as described in data module manual for external standard quantitation.

Discussion

Method Development

Development of this procedure was preceded by establishment of some general performance criteria for each step. The initial requirement was for an extraction solvent that would not require either time-consuming partition techniques or volume reduction before chromatographic purification.

An elution series had to be developed in which aflatoxins would be adsorbed onto the silica cartridge and retained while less polar interferences were eluted. Quantitative elution with less than 20 mL solvent was desired to avoid volume reduction and transfer to a smaller container. Because the method would be applied to heavily contaminated materials, the capacity of the silica cartridge had to be investigated. Fifteen mL was chosen as the maximum volume for application to cartridge to avoid contact between

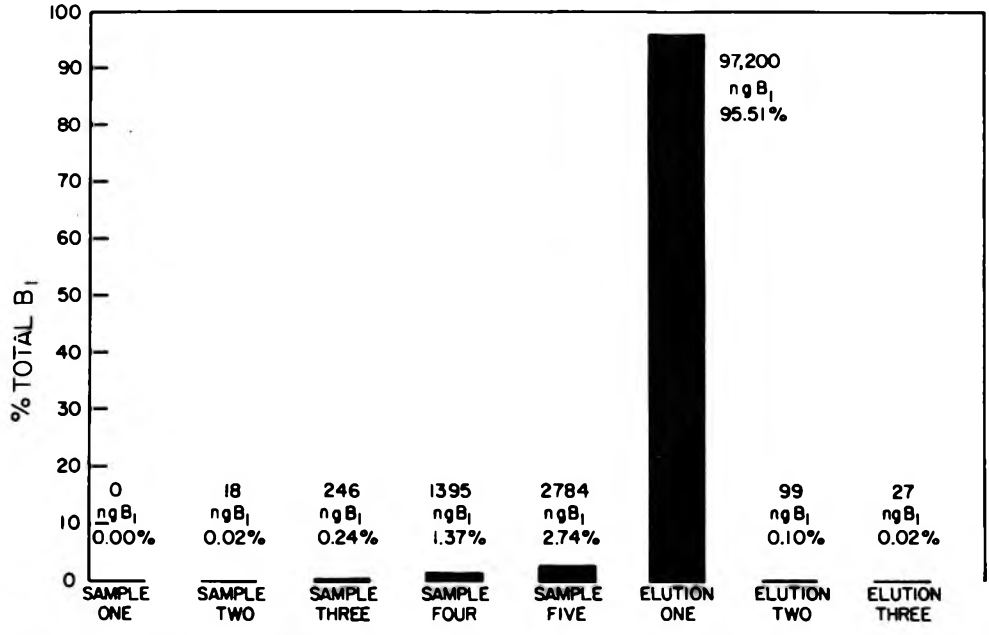

Figure 1. Elution profile of 5 successive portions of sample mixture and 3 successive portions of hexane-acetone (1 + 1) elution solvent applied to a silica cartridge. Sample portions were chloroform extract of corn mixed with hexane (1 + 1). Each sample portion contained about 20 μg B_1; no rinse solution was applied.

solution and plunger of the syringe. Silica cartridges are wet with hexane before sample is applied in accordance with suggestions made by the manufacturer.

A reliable procedure for the derivatization of aflatoxins B_1 and G_1 was needed. We decided that the sample residue should be completely dissolved in the derivatization reagent with no phase separation.

LC separation and fluorometric determination would have to yield consistently low variation in retention times and peak areas for replicates of both standards and samples. Phase separation, evident as small droplets of oily liquid appearing in the redissolved extract, should not be present and should not be masked by vortex mixing (9); volume changes due to phase separation would interfere with the accuracy of the quantitative determination.

Extraction Solvent.—Chloroform was chosen as the extraction solvent because addition of an equal volume of hexane to the chloroform extract reduced polarity of the solution sufficiently to allow retention of toxins on the silica cartridge. Aqueous methanol has been reported to achieve a more efficient extraction but requires that toxins be partitioned into organic phase, the solvent evaporated, and the extract redissolved before column cleanup (5, 11, 13).

Cartridge Chromatography.—Preliminary experiments using detection by visualization of aflatoxins on developed thin layer plates under longwave (365 nm) UV light revealed that aflatoxin was completely retained on the cartridge when the chloroform extract was mixed 1:1 with hexane, and that aflatoxin was quantitatively eluted by a 1:1 mixture of hexane–acetone (data not shown).

Application of Sample.—Experiments were conducted to determine the number of 15 mL portions of the chloroform extract–hexane mixture that could be applied before aflatoxins began to elute from the cartridge. Results of application of 5 successive 15 mL portions of sample mixture are shown in Figure 1. The sample used was from a 1 kg sample of finely ground corn spiked with about 1–2 g rice culture of *Aspergillus parasiticus* NRRL 2999 to contain a level of about 15 mg B_1/kg. Each sample portion contained about 20.4 μg B_1. Sample portions that had been passed through a cartridge were evaporated to dryness, derivatized, and quantitated by LC. Cartridges were quite retentive for aflatoxin. Over 95% of the toxin was retained on the cartridge even after the application of five 15 mL portions containing a total of about 102 μg B_1. A single 15 mL portion, equivalent to 1.5 g starting material, is sufficient for quantitative determi-

Table 1. Precision of retention times and peak areas for aflatoxins separated on 5 and 10 μm C_{18} columns[a]

Statistic	G_{2a}	B_{2a}	G_2	B_2
	Separation on 5 μm Column[b]			
Mean retention time, min	5.22	7.23	11.05	17.37
Std dev.	0.07	0.11	0.19	0.33
Coeff. of var., %	1.44	1.55	1.72	1.90
Mean peak area, mV·s	73 420	192 917	80 018	111 282
Std dev.	832	2003	765	1173
Coeff. of var., %	1.13	1.04	0.96	1.05
	Separation on 10 μm Column[c]			
Mean retention time, min	5.07	6.91	11.69	17.90
Std dev.	0.04	0.05	0.08	0.13
Coeff. of var., %	0.81	0.77	0.65	0.70
Mean peak area, mV·s	78 572	204 247	107 690	148 874
Std dev.	1155	1966	656	998
Coeff. of var., %	1.47	0.96	0.61	0.67

[a] Retention time and peak areas were determined electronically. Twelve 2 μL injections were made of standard containing 5.0 ng G_1 (as G_{2a})/μL, 10.0 ng B_1 (as B_{2a})/μL, 5.0 ng G_2/μL, and 5.0 ng B_2/μL.
[b] Mobile phase was 2% acetic acid, 10% 2-propanol, and 10% acetonitrile in water at flow rate of 1.5 mL/min.
[c] Mobile phase was 7% acetic acid, 7% 2-propanol, and 7% acetonitrile in water at flow rate of 2.0 mL/min.

nation with the sensitive fluorescence detection system used. The limit of detection was about 0.1 ng B_1 under the conditions used.

Rinse Solution.—A mixture of hexane and chloroform (3 + 1) eluted nonpolar fluorescent interferences and "oils" from the cartridge without eluting the aflatoxin. Even after application of five 15 mL portions of rinse solution over 99% of the aflatoxin (about 22.4 μg B_1) was retained on the silica cartridge. Observation of the extracts after evaporation of solvent showed that two 15 mL portions are sufficient for removing oils which induced phase separation when the sample was derivatized or redissolved for LC determination. TLC of the extracts revealed that some fluorescent interferences, less polar than aflatoxin B_1, were eluted by the application of the two 15 mL portions of rinse solution.

Quantitative Elution.—A single 15 mL portion of hexane–acetone (1 + 1) quantitatively eluted the aflatoxins from the cartridge. A corn extract containing 23.16 μg B_1 (15.44 mg B_1/kg) was applied. The cartridge was washed with two 15 mL portions of rinse solution and then 5 successive portions of elution solvent. No detectable aflatoxin was eluted before application of the elution solvent (detection limit about 0.12 ng B_1 [as B_{2a}] under conditions used). All the detectable aflatoxin was eluted with the first 15 mL portion of elution solvent.

Maximum Cartridge Capacity.—The maximum capacity of the silica cartridge was not determined per se; however, when 15 mL of an extract of 50 g corn sample spiked with rice culture to contain 165.33 mg B_1/kg was applied to the cartridge (248 μg B_1), over 99.9% of the aflatoxin applied remained on the cartridge through the rinsing step to be eluted in the first portion of elution solvent. Inoculated field corn analyzed in this laboratory has never exceeded 30 mg B_1/kg, and inoculated corn produced in growth chambers has never exceeded 150 mg B_1/kg in this laboratory.

Derivatization.—The volume (1.5 mL) of TFA–water allowed samples to be completely dissolved during the derivatization step, which was not the case with other methods tested in this laboratory (9, 13–16). Addition of 10% water to trifluoroacetic acid expedited hemiacetal formation. Derivatization reagent was evaporated to avoid the introduction of a corrosive halogenated compound into the liquid chromatographic system and to avoid use of TFA outside of the fume hood. Total time required for volume reduction was less than 30 min.

LC Separation and Quantitation.—Aflatoxins G_{2a}, B_{2a}, G_2, and B_2 dissolved in acetonitrile were re-

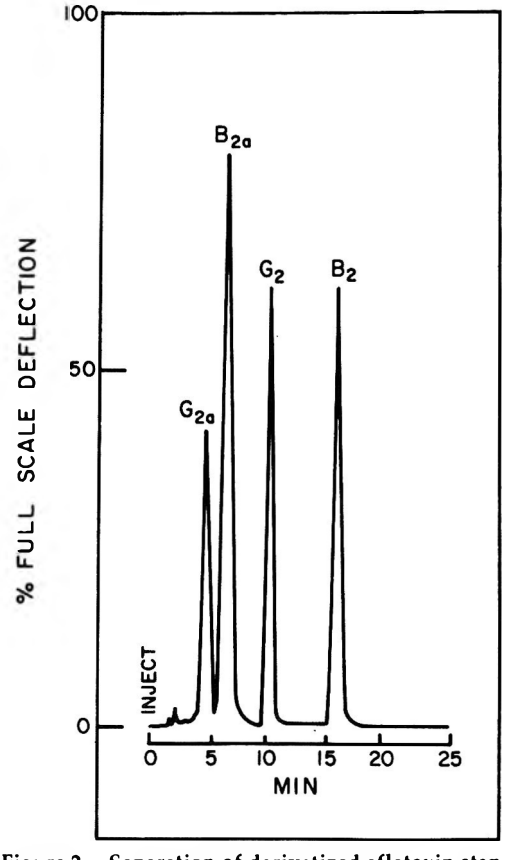

Figure 2. Separation of derivatized aflatoxin standards on 5 μm C_{18} column: 30 μL injection containing 4.5 ng G_1 (as G_{2a}), 9.0 ng B_1 (as B_{2a}), 4.5 ng G_2, and 4.5 ng B_2.

solved at baseline (in less than 25 min) using a 5 μm C_{18} column and a mobile phase of 2% acetic acid, 10% acetonitrile, and 10% 2-propanol in water at a flow rate of 1.5 mL/min. Retention times were 5.22 min for aflatoxin G_{2a}, 7.23 min for aflatoxin B_{2a}, 11.05 min for aflatoxin G_2, and 17.37 min for aflatoxin B_2 (Table 1). Separation of aflatoxins using a 10 μm C_{18} column was accomplished using a mobile phase of 7% acetic acid, 7% acetonitrile, and 7% 2-propanol in water at a flow rate of 2 mL/min. Analysis was complete within 25 min with retention times of 5.07 min for aflatoxin G_{2a}, 6.91 min for aflatoxin B_{2a}, 11.69 min for aflatoxin G_2 and 17.90 min for aflatoxin B_2 (Table 1). No interfering peaks were detected even in highly contaminated samples due to the high resolution of the column and the selectivity of fluorescence detection. Representative chromatograms are shown in Figures 2, 3, and 4. For routine analysis of heavily contaminated material, the detection/quantitation system is set to provide results in the 0.5 to 10.0 mg B_1/kg range. If aflatoxins are not present within this range, the detector is reattenuated and the data module is recalibrated with an appropriate standard. Samples may be quantitated at levels as low as 10 μg B_1/kg by this procedure or to 0.5 μg B_1/kg by using larger injection volumes and/or smaller sample volumes.

Comparison with Official Method.—When the cartridge method was compared with AOAC official method I (CB method) at a level of about 50 μg B_1/kg in naturally contaminated corn, the results of the 2 methods were in good agreement (Table 2). At a level of about 10 mg B_1/kg, however, the cartridge method yielded a substantially higher mean and lower variance than the official method. Chloroform extracts at each level were filtered, pooled, and mixed well. Five 50 mL portions were drawn for analysis by the

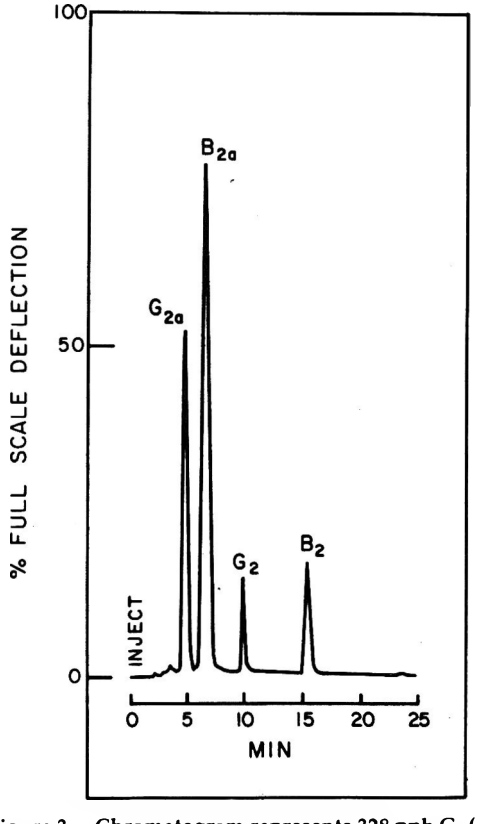

Figure 3. Chromatogram represents 328 ppb G_1 (as G_{2a}), 512 ppb B_1 (as B_{2a}), 55 ppb G_2, and 73 ppb B_2 in naturally contaminated corn (5 μL injection). Toxins were separated on 5 μm C_{18} column.

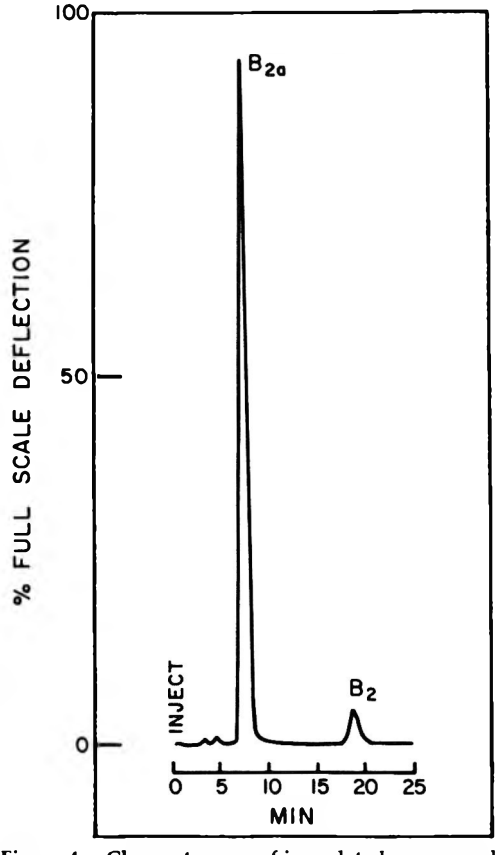

Figure 4. Chromatogram of inoculated corn sample containing 5.6 ppm B_1 (as B_{2a}) and 0.2 ppm B_2 (20 μL injection). Toxins were separated on 10 μm C_{18} column.

official method and five 25 mL portions were drawn for the cartridge method.

Precision Study.—Three 50 g samples of naturally contaminated corn were extracted, filtered, and combined. A pooled extract was used so that variation would be due to procedure rather than to extraction or variation in corn samples. A 250 mL portion of the pooled extracts was mixed with 250 mL hexane, and thirty 15 mL aliquots of the extract–hexane mixture were processed by the cartridge method. The values obtained for aflatoxin B_1 and B_2 are listed in Table 3. The coefficient of variation was acceptably low (8.46%) at a mean value of 1066 μg B_1/kg.

Table 2. Comparison of cartridge method with official method I at 2 levels [a]

Sample	Method	B_1, μg/kg	Std error, μg/kg	CV, %
1 [b]	offic.	54.0	1.13	4.17
	cartr.	58.0	0.29	0.87
2 [c]	offic.	4490	360	17.82
	cartr.	10 090	133	2.94

[a] Chloroform extracts at each level were pooled, and 5 replicate samples were drawn for each method; values were determined by LC separation on 5 μm C_{18} column and fluorescence detection.

[b] Naturally contaminated corn.

[c] Corn spiked with *Aspergillus parasiticus* rice culture.

Table 3. Precision study of cartridge method [a]

Replicate	μg B_1/kg	μg B_2/kg
1	846	172
2	942	172
3	946	171
4	1135	174
5	1140	175
6	924	173
7	1101	169
8	1185	171
9	1111	174
10	1006	170
11	935	170
12	1125	175
13	1148	177
14	1132	175
15	1012	171
16	1010	170
17	1095	174
18	1146	173
19	992	176
20	1002	174
21	1072	175
22	1125	177
23	1132	176
24	1175	174
25	1122	172
26	1183	169
27	1109	178
28	1086	173
29	1096	172
30	956	172
Mean	1066	173
Std error	16.5	0.44
CV (%)	8.46	1.40

[a] Values were determined by LC separation on 10 μm C_{18} column with fluorescence detection.

Acknowledgments

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Five-Year Study of Mycotoxins in Virginia Wheat and Dent Corn

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Every year during the 5-year period 1976-1980, approximately 100 samples each of corn and wheat from trucks delivering the grains at elevators in Virginia were collected by personnel of the Federal Grain Inspection Service and shipped to NRRC. Samples were analyzed as soon as possible for aflatoxin, zearalenone, and ochratoxin A. The 3 mycotoxins were not detected in any wheat sample. Zearalenone and ochratoxin A were not found in any corn sample; however, aflatoxin was detected in at least 25% of the corn samples from every crop year. In 1976-1980, the incidence of aflatoxin at levels of 20 ng/g or more (the Food and Drug Administration guideline) ranged from 18 to 61%; aflatoxin incidence above 100 ng/g was 5-29%. The average aflatoxin levels in corn samples collected in the 5 years varied from 21 to 137 ng/g. Moisture content of the samples was not determined, so aflatoxin levels given may be higher than they were at harvest. However, there are obviously differences from year to year. In freshly harvested corn samples collected by fieldmen of the Statistical Reporting Service in yield surveys in 1978 and 1979, aflatoxin incidence above the FDA guideline was 10 and 13%, and above 100 ng/g was 4 and 7%. The average aflatoxin level in all samples collected in 1978 was 13 ng/g and in 1979, 36 ng/g. Some aflatoxin can be expected yearly in Virginia corn, but the incidence and levels vary from year to year.

In 1975, the occurrence of aflatoxin, zearalenone, and ochratoxin A was studied in wheat grown in areas where aflatoxin has been a problem (1). Of 42 wheat samples collected in Virginia, 19 had zearalenone, but none had aflatoxin or ochratoxin A. Virginia weather was unusually cold and rainy in 1975; wheat fields were infected with *Fusarium* species that produce zearalenone. Graders of the Federal Grain Inspection Service (FGIS) observed a large number of "tombstone" wheat samples with the grey pallor characteristic of wheat invaded by *Fusarium*. *Fusarium* outbreaks in wheat ("scabby wheat") are said to be associated with the type of weather conditions experienced in 1975, and these conditions might occur about once every 5 years in the weather cycles (1). Because of the high zearalenone incidence, it was decided to study wheat over a period of years to determine how often such outbreaks could be expected. At the same time, wheat would be analyzed for aflatoxin and ochratoxin A, and the investigation would be extended to corn. A reliable screening procedure is available to detect the 3 mycotoxins in agricultural commodities (2).

We now are reporting the results of a 5-year study of wheat and corn grown in Virginia for the occurrence of aflatoxin, zearalenone, and ochratoxin A. The samples were collected by FGIS personnel. We also are reporting the aflatoxin occurrence in freshly harvested corn samples collected by fieldmen of the Statistical Reporting Service (SRS) in 1978 and 1979.

Materials and Methods

Sample Collection and Handling

About 100 samples each (ca 1.3 kg) of wheat and shelled dent corn were collected each year during the 5-year period 1976-1980 by FGIS personnel from farm trucks delivering at Virginia elevators. The corn and wheat were grown in the same year that samples were collected, and the county in which they were grown was recorded. The samples were shipped to Peoria as soon as collected and were assayed upon arrival in Peoria. Moisture levels were not determined.

In 1978 and 1979, one 15 ft row of mature dent corn was harvested by SRS fieldmen from each field to obtain about 100 samples of shucked ear corn. The collection was made as SRS personnel were conducting yield surveys and was representative of the corn grown in Virginia in that year. Samples were shipped to Peoria in corrugated cardboard boxes surrounded by newspapers and were in transit about 7 days. When received in Peoria, they were shelled and dried (overnight 80°C). The shelled dried samples averaged 2.2 kg.

Preparation of Subsamples for Analysis

Each sample of shelled corn was coarsely ground in a Straub disc mill and then finely ground to pass a No. 20 sieve in a 6 in. Raymond

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

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hammer mill fitted with a screen containing 1/8 in. perforations. Wheat samples could be ground rapidly in the Raymond hammer mill to pass a No. 20 sieve without being coarsely ground in the disc mill first. Each finely ground sample of wheat or corn was blended 15 min in a Hobart planetary mixer.

Analysis for Mycotoxins

Subsamples (50 g) of corn or wheat collected by FGIS personnel were analyzed by the Eppley procedure for aflatoxin, zearalenone, and ochratoxin A (2). Samples are extracted with chloroform–water and extract aliquots representing 10 g of the subsample are placed on a silica gel column. Zearalenone, aflatoxin, and ochratoxin A are eluted separately from the column and eluates are prepared for thin layer chromatography (TLC). Solvents for TLC on Absorbosil-1 plates are as follows: for zearalenone, ethanol–chloroform (5 + 95); for aflatoxin, water–acetone–chloroform (0.5 + 10 + 90); and for ochratoxin A, glacial acetic acid–benzene (10 + 90). The detection limits of the analysis as carried out were 100 ng/g for zearalenone, 1–3 ng/g for aflatoxins, and 30 ng/g for ochratoxin A. If a sample was positive for ochratoxin A by TLC, the result was tested by the confirmatory test approved by the Association of Official Analytical Chemists (AOAC) (3). If a sample contained aflatoxins, levels were determined by the method designated as the CB (Contaminants Branch) method approved for corn by both the AOAC (3) and the American Association of Cereal Chemists (AACC) (4).

Subsamples (50 g) from corn collected by SRS fieldmen were assayed for aflatoxins by the CB method approved for corn by the AOAC (3) and AACC (4).

Results and Discussion

Zearalenone, aflatoxins, and ochratoxin A were not detected in any of the wheat samples collected in the period 1976–1980. The high incidence (45%) of zearalenone in 1975 wheat from Virginia must have been unusual (1). Zearalenone was not detected in any of the corn samples. Some of the extracts of corn partially purified on silica gel columns by the Eppley procedure (2) contained a fluorescing substance with the same R_f value as ochratoxin A on TLC plates. However, the test to confirm the identity of ochratoxin A by formation of the ethyl ester was negative (3). False positive tests for ochratoxin A in corn have been observed previously (5, 6).

Aflatoxins were detected in corn samples collected in every year by FGIS personnel (Table 1). The percent incidence of samples with detectable aflatoxin varied from 29% (1979) to 82% (1980). The incidence of samples with aflatoxin levels equal to or greater than 20 ng/g ranged from 18% (1979) to 61% (1980). Average aflatoxin levels in all samples collected in a year during the period 1976–1980 were between 21 ng/g in 1978 and 137 ng/g in 1980. In 1980, the aflatoxin incidence in the FGIS samples above the guidelines was unusually high—61%. Of the 1980 samples, 29% had more than 100 ng/g. Aflatoxin levels in corn were high in southeastern corn in 1980 (7). In fact, the situation was so serious that the Food and Drug Administration (FDA) granted limited exemptions to the states of North Carolina and South Carolina and the Commonwealth of Virginia from its prohibition of interstate shipment and blending of corn containing aflatoxin in excess of the agency's action level of 20 ng/g (8). The FDA allowed corn containing up to 100 ng/g to be shipped in interstate commerce under plans developed by the 3 states and approved by the FDA. The exemption applied only to corn from the crop year 1980. Some toxin could have formed after samples were collected, because some of the samples were noticeably damp when received (9).

Corn samples were collected by the FGIS in 53 counties in Virginia in the 5-year period. However, from some counties samples were received in only 1 or 2 years. There were 8 counties from which samples were collected in every year, 1976–1980. Inspection of aflatoxin levels in corn samples in the 18 counties where 10–55 samples were collected in the 5-year period did not reveal obvious differences in aflatoxin occurrence among counties. There were not enough samples collected in each county to evaluate statistically the likelihood of an aflatoxin outbreak in any one county or area.

At first, aflatoxin levels and incidences appeared to be lower in freshly harvested corn samples collected by SRS fieldmen than in the shelled samples collected by FGIS personnel from trucks delivering at elevators (Table 1). In 1978, aflatoxin incidence in FGIS corn samples was 36% compared with 12% in SRS corn samples. However, in 1979, the toxin incidence was 29% in FGIS samples compared with 21% in SRS corn samples. The average aflatoxin level in SRS samples was 13 ng/g in 1978 and 36 ng/g in 1979; in FGIS samples, 21 ng/g in 1978 and 34 ng/g in 1979.

Of the 3 mycotoxins studied in wheat and

Table 1. Aflatoxin levels in dent corn grown in Virginia, 1976–1980

	Collected from trucks by FGIS										Collected at harvest by SRS			
	1976		1977		1978		1979		1980		1978		1979	
Total aflatoxin, ng/g	No. of samples	(%)	No. of samples	(%)	No. of samples	(%)	No. of samples	(%)	No. of samples	(%)	No. of samples	(%)	No. of samples	(%)
ND[a]	77	(63)	52	(51)	63	(64)	81	(71)	18	(18)	79	(88)	93	(79)
<20	13	(10)	17	(17)	10	(10)	13	(11)	20	(20)	2	(2)	9	(8)
20–100	21	(17)	18	(18)	21	(21)	8	(7)	32	(32)	5	(6)	7	(6)
101–500	9	(7)	10	(10)	5	(5)	10	(9)	26	(26)	4	(4)	7	(6)
501–1000	1	(1)	1	(1)	—	—	2	(2)	1	(1)	—	—	—	—
>1000	2	(2)	3	(3)	—	—	—	—	2	2	—	—	1	1
Total	123		101		99		114		99		90		117	
% Incidence	37		49		36		29		82		12		21	
% ≥ 20 ng/g	27		32		26		18		61		10		13	
% > 100 ng/g	10		14		5		11		29		4		7	
Av. level (ng/g), all samples	48		91		21		34		137		13		36	
Av. level (ng/g), pos. samples	130		187		58		118		167		110		176	

[a] ND = not detected.

corn, only aflatoxin was identified as a continuing problem in corn. From our investigations in 1975 wheat, we learned that zearalenone could cause problems when weather conditions were favorable for *Fusarium* invasions of wheat (scabby wheat). However, the fact that zearalenone was not detected in wheat harvested in 1976–1980 indicates that one outbreak of scabby wheat does not necessarily lead to infestation of crops in subsequent years. This information became more important in 1982, when scab-damaged wheat from a few areas was found to contain deoxynivalenol and to a lesser degree zearalenone—2 mycotoxins produced by *Fusarium* (10). The weather was unusually rainy in the areas where scabby wheat occurred in 1982.

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Rapid Detection of *Fusarium* Mycotoxins in Grains by Quadrupole Mass Spectrometry/Mass Spectrometry

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The *Fusarium* mycotoxins deoxynivalenol (DON) and zearalenone can be detected rapidly in a crude extract of a grain matrix by quadrupole mass spectrometry/mass spectrometric analysis of the collisionally activated dissociation (CAD) daughter fragments of the protonated molecule. Detection limits were as low as 0.1 ppm. The 2 toxins were detected in a single analysis. Monitoring the CAD daughters of the negative ion m/z 248 of DON eliminated most interferences from the grain matrix and produced daughter spectra identical to those produced from an authentic standard.

Under certain conditions, *Fusarium* molds invade cereal grains in the field or in storage. These molds produce several toxic secondary metabolites (mycotoxins) that contaminate the grains. When these contaminated grains are fed to susceptible animals, they can cause physiological disorders of economic significance. Among these mycotoxins are the trichothecene deoxynivalenol (DON) (3,7,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one), also known as vomitoxin, which has been associated with feed refusal and animal disorders known as fusariotoxicoses (1), and the estrogen zearalenone [6-(10-hydroxy- 6 -oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone], which causes hyperestrogenism in swine (2).

Both DON and zearalenone have been found in corn (3-5), barley (6), and wheat (7). After substantial workup, these mycotoxins have been identified and quantitated by gas chromatography and gas chromatography–mass spectrometry of the trimethylsilyl or heptafluorobutyrate derivatives (3, 7) and by electron capture (EC) GC (7).

The rather new technique of tandem mass spectrometry, or mass spectrometry/mass spectrometry (MS/MS), has generated considerable interest as a method to analyze for target compound(s) in a crude matrix of organic compounds. MS/MS uses one stage of mass separation to select the compound of interest from the matrix (usually as the molecular ion, protonated molecular ion, or molecular anion) and a second stage for analysis after a collisionally activated dissociation (CAD) by collision with a target gas. The wide variety of emerging instruments being developed for MS/MS studies has been reviewed (8, 9). We now report detection by MS/MS of zearalenone and DON at below 1 ppm concentrations directly from a solvent extract of the grain matrix and without sample derivatization or sample cleanup. Selection of several parent/daughter experiments allows detection of both mycotoxins in a single analysis.

Experimental

A Finnigan 4535/TSQ triple quadrupole mass spectrometer equipped with pulsed positive-negative ion chemical ionization was used in MS/MS analysis. Isobutane was the reagent gas or buffer gas (negative ion) at a pressure of 0.25 torr. Source temperature was maintained at 140°C and electron energy was 70 eV. Argon was the target gas in the Q_2 collision cell for CAD experiments. The pressure of Q_2 was set at 1.5 mtorr as measured by the Hastings gauge. This gave a pressure of ca 2.5×10^{-5} torr in the analyzer region of the mass spectrometer. Collision energy of the CAD in Q_2 measured as the axial dc offset potential of Q_2 was +20 V for negative ion experiments and −12 to −20 V for positive ion experiments. Because the daughter ions produced in low-energy CAD experiments with quadrupole MS/MS are highly sensitive to both target gas pressure and collision energy, careful setting of instrument parameters is required for reproducibility.

Samples were introduced via the direct insertion probe, which was heated by the ballistic ramp heater set at a dial reading of 3.5. This setting heated the probe to above 400°C in about 4 min. Under these conditions, the mycotoxins analyzed eluted from the probe as a peak in about 2½ min. Data acquisition and mass spectrometer control were accomplished by an Incos 2300 data system with specialized software that controlled

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scanning and mass setting parameters for the quadrupoles in MS/MS experiments. Q_1 was set to alternatively pass the protonated molecule at m/z 319 for zearalenone, at m/z 297 for DON, or the negative ion chemical ionization (NICI) MS fragment at m/z 248 for DON. Full scans of the CAD daughter fragments were recorded from Q_3. Alternating the parent ion focused into Q_2 for CAD allowed simultaneous elution profiles for both DON and zearalenone to be recorded in a single analysis.

Sample Preparation

Ground and blended 50 g samples of grain (corn, wheat, oats, and rice) were extracted in 250 mL of one of 4 solvents [$CHCl_3$–ethanol (80 + 20); $CHCl_3$–water (250 + 25); CH_3CN; or methanol–water (1 + 1)] for 30 min on a wrist-action shaker. A 50 mL portion of the filtered extract (10 g equivalent) taken to dryness on a rotary evaporator was transferred in $CHCl_3$ to a Teflon-lined screw-cap vial dried under nitrogen, and the residue was dissolved in 0.5 mL $CHCl_3$.

A 1–5 μL portion of the sample (equivalent to 20–100 mg grain) was put into a probe sample cup, allowed to evaporate to dryness, and then analyzed by MS/MS.

For determination of detection limits, mycotoxin-free grain samples were spiked at 0.1, 0.5, 1.0, and 2.0 ppm with DON and/or zearalenone and extracted as above. Also, blank samples were spiked to a 1.0 ppm toxin concentration after extraction. Recoveries of toxins were determined on spiked samples, using the method of Shotwell et al. (10) for zearalenone and a modified Scott et al. (7) procedure for DON. Zearalenone and DON in extracts partially purified by silica gel column chromatography were quantitated by densitometry and gas chromatography with electron capture detection, respectively.

Results and Discussion

The isobutane CI-MS of zearalenone (Figure 1) shows predominantly the protonated molecule at m/z 319. Little fragmentation is observed. Daughter spectra of m/z 319 varied considerably with the collision energy (Q_2 offset voltage) (Figure 2). At low offset voltages, relatively little fragmentation occurs with m/z 319, and losses of 18 and 36 dominate the spectrum. At moderate Q_2 offsets (about −12 V), considerably more fragmentation occurs. The base peak of the daughter spectrum is m/z 187 and the size of the m/z 319 parent peak is reduced to about 10% of the low energy daughter abundance. At higher energies, the parent peak virtually disappears and lower mass daughters dominate the spectrum.

The amount of fragmentation in the daughter

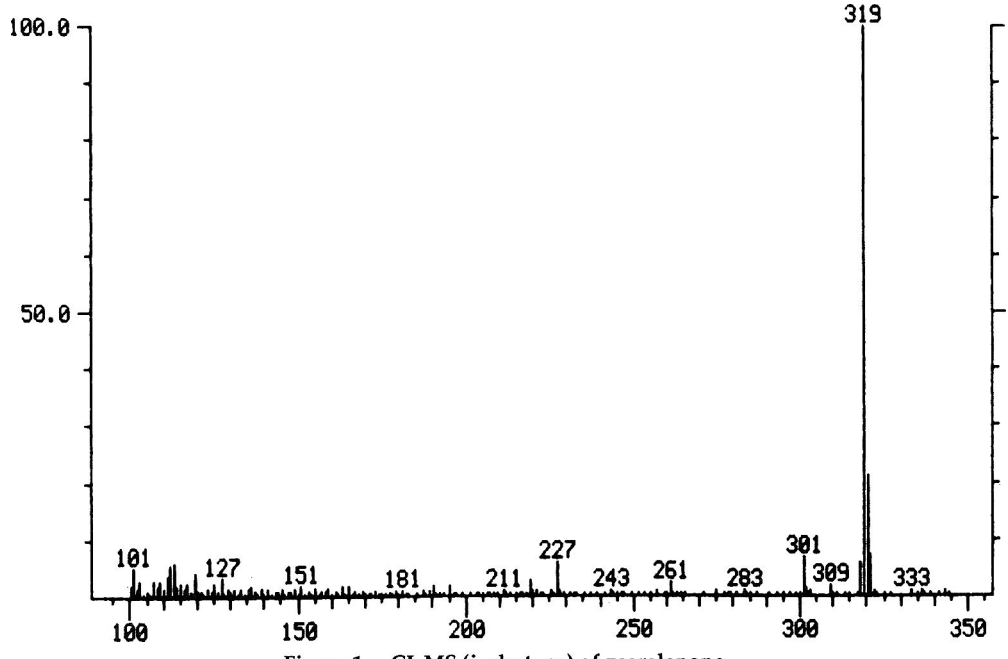

Figure 1. CI-MS (isobutane) of zearalenone.

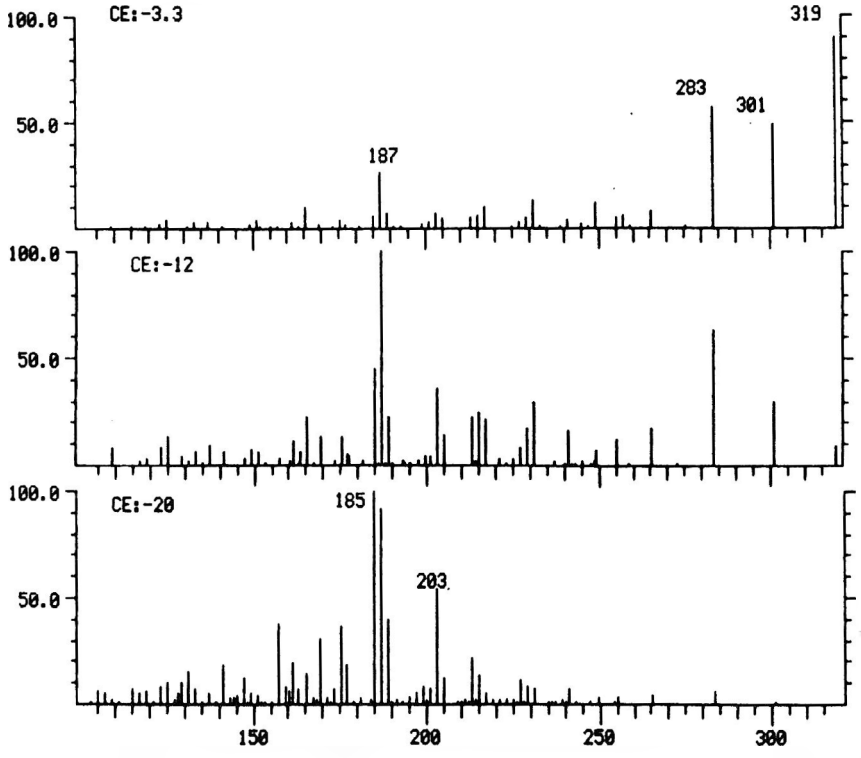

Figure 2. Argon CAD daughters of m/z 319 of zearalenone at several collision energies at (−3, −12, and −20 V) 1.5 mtorr.

spectra is also a function of target gas pressure. Increasing the target gas pressure from 1.5 to 3.0 mtorr increased the fragmentation at lower offset voltages. A target gas pressure of 1.5 mtorr and an offset voltage of −12 V were chosen as optimum for zearalenone daughter experiments because good sensitivity, along with large daughter fragments, were produced. It was felt that a more abundant, lower mass fragment, such as the m/z 187 daughter, would be much less likely to be a prominent daughter of any interfering components in the matrix, than m/z 301 and m/z 283, which could arise from water losses from an m/z 319 parent. Ten nanograms of zearalenone produced good daughter spectra from m/z 319 under these Q_2 pressure and offset voltages. Figure 3 shows the MS/MS daughter spectrum of a $CHCl_3$–water extract of clean, zearalenone-free corn that was spiked at 1.0 ppm with zearalenone (Figure 3a) vs the spectrum of the clean corn extract (Figure 3b). These spectra were obtained from MS/MS of an aliquot of the corn extract equivalent to 100 mg corn. The daughter spectrum of the spiked corn is virtually identical to the m/z 319 daughter spectrum of the pure toxin. At the 1 ppm level, interference from the matrix is minimal in corn, wheat, rice, and oats extracts. At lower levels, the matrix m/z 319 daughter signals become large relative to the zearalenone m/z 319 daughter signals. However, no signals are observed for m/z 319 daughters at m/z 185, 187, or 203 in zearalenone-free samples. Zearalenone was detectable in all spiked and naturally contaminated grain extracts as low as 0.1 ppm with no sample cleanup.

The CI-MS of DON (Figure 4) showed more fragmentation than the CI-MS of zearalenone. The protonated molecule at m/z 297 is the base peak in the spectrum, but its percentage of the total ionization is considerably less than for zearalenone. This meant that daughter scans for m/z 297 from 10 ng DON were somewhat less intense than daughter scans of m/z 319 from 10 ng zearalenone. The m/z 297 daughter spectrum at Q_2 pressure of 1.5 mtorr and offset voltage of −12 V is shown in Figure 5. The daughters observed are similar to the fragments in the CI-MS of DON (Figure 4). The major m/z 297 daughter fragments at m/z 175, 189, 203, 219, 231,

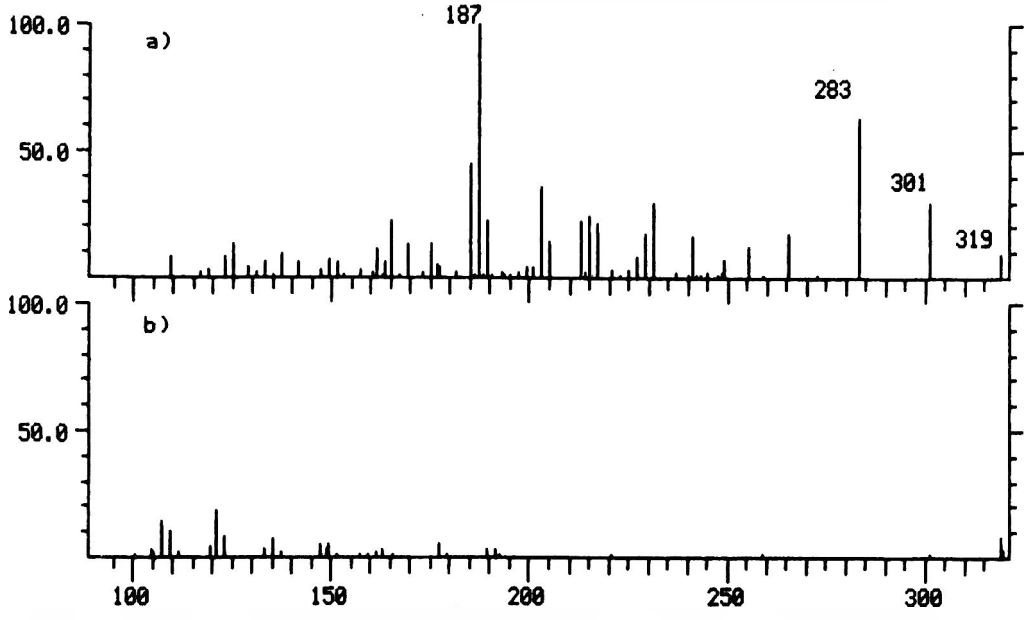

Figure 3. Comparison of CAD daughter spectra for (a) m/z 319 from extract of corn spiked with 1.0 ppm zearalenone, and (b) m/z 319 from extract of mycotoxin-free corn.

and 249 are all important fragments observed in the CI spectrum, but the ratios are different. When the daughter spectra of m/z 297 were recorded for an aliquot equivalent to 100 mg corn, intense fragments were observed, which indicates the presence of substantial m/z 297 ions in the blank matrix. However, examination of the daughter spectrum reveals that no daughter fragments are observed at m/z 175, 203, or 249, all of which are intense fragments from the

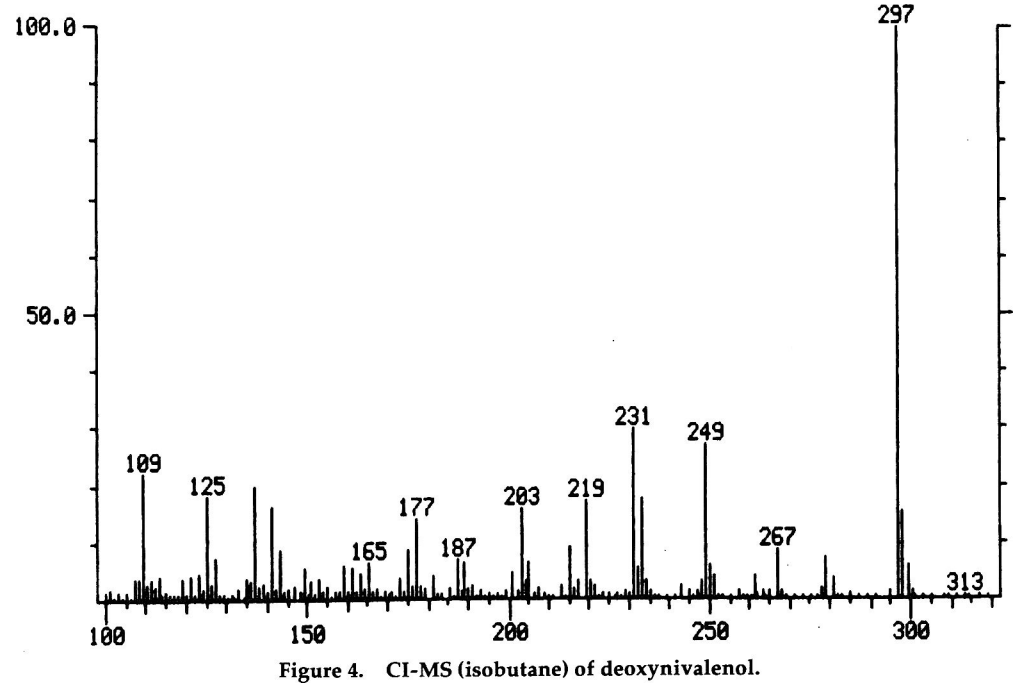

Figure 4. CI-MS (isobutane) of deoxynivalenol.

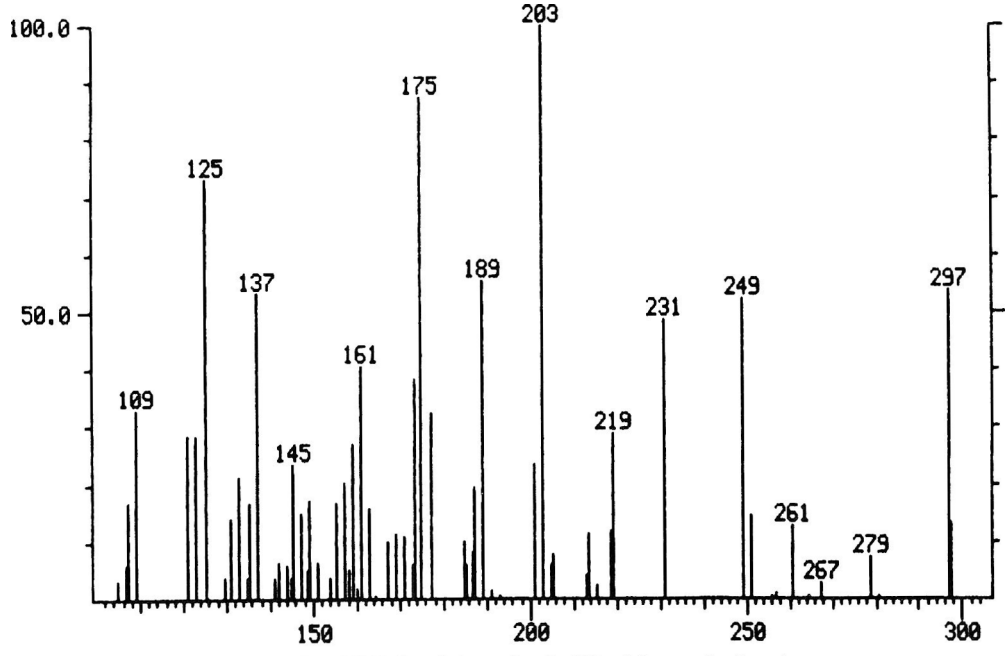

Figure 5. CAD daughters of m/z 297 of deoxynivalenol.

protonated molecule of DON (Figure 5). Daughter spectra of a spiked corn matrix (1.0 ppm) and a mycotoxin-free corn matrix are shown in Figure 6. Plots of the daughters at m/z 203, 231, and 249 for the spiked matrix and the blank matrix demonstrate that the DON can still be seen even with the interferences from the matrix. Similarly, the mycotoxin-free matrixes from wheat, oats, and rice extracts showed intense m/z 297 daughter spectra but again no

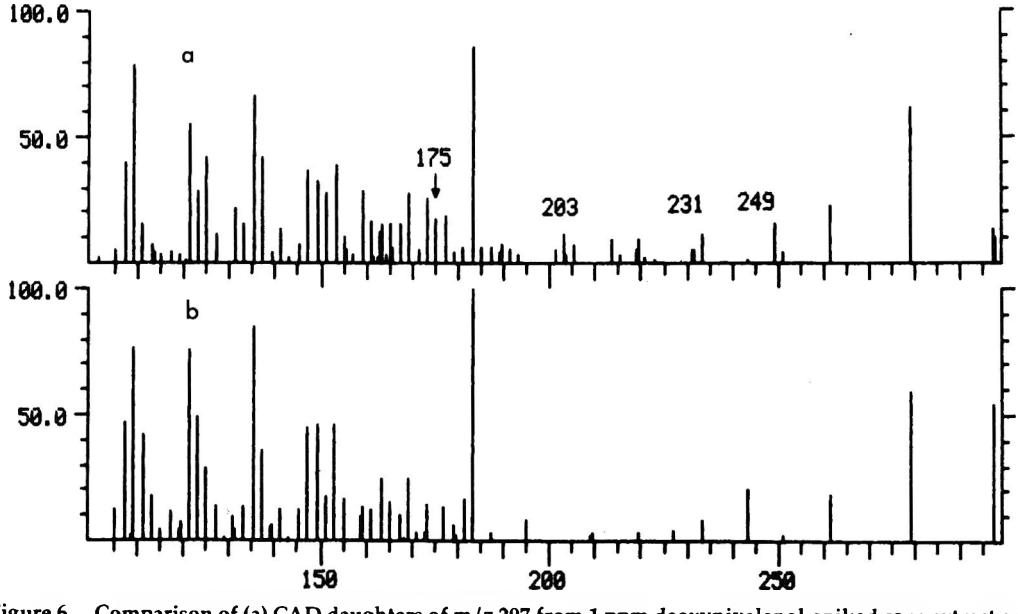

Figure 6. Comparison of (a) CAD daughters of m/z 297 from 1 ppm deoxynivalenol-spiked corn extract and (b) m/z 297 from extract of mycotoxin-free corn.

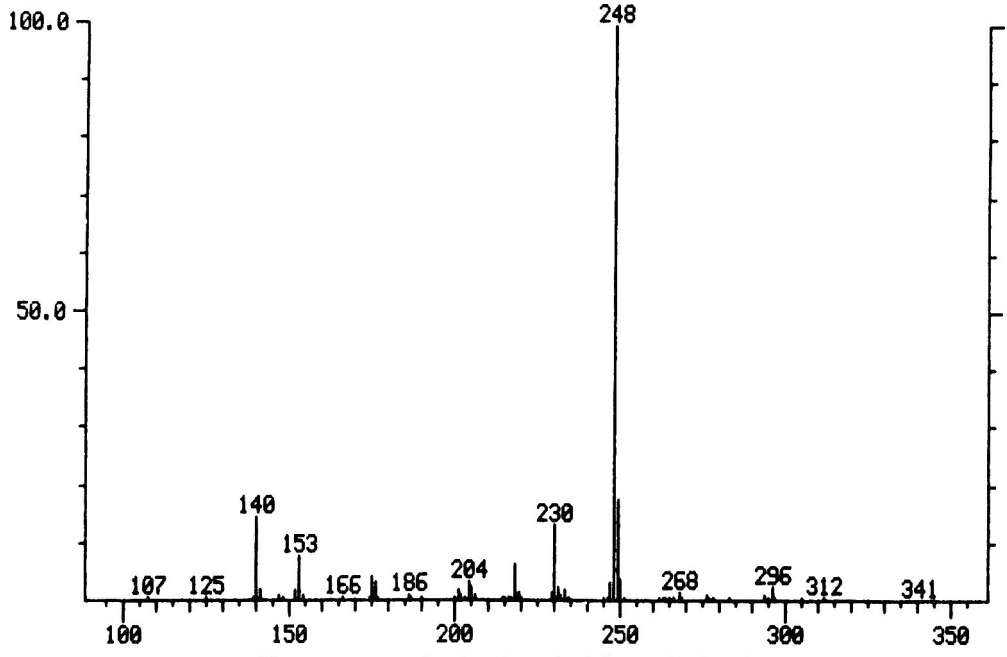

Figure 7. Negative ion CI-MS of deoxynivalenol.

daughter at the m/z values of m/z 203, 231, and 249, making detection of DON at 1 ppm possible by daughters of m/z 297. In the NICI-MS mode with isobutane, a small signal for the molecular anion of DON at m/z 296 is observed. The base peak in the spectrum, m/z 248, arises from the loss of 48 (Figure 7). Presumably, this is the loss of $H_2C{=}O + H_2O$. This signal is on the order of 100 times as intense as the protonated molecule observed in the positive ion spectrum under the same conditions. Daughter spectra of m/z 248 from 10 ng DON (Figure 8) and from a mycotoxin-free corn extract spiked with 1.0 ppm DON were nearly identical. Very little m/z 248 is in the blank matrix, and no signal at the major DON daughters at m/z 147 and 175 is observed in the blank. Similarly, blank matrixes of wheat, oats, and rice showed negligible interference at m/z 248. Thus DON could be easily detected, because it produces nearly identical MS/MS daughter spectra of m/z 248 as the pure component when its presence in the matrix was only at the 0.5 ppm level. The positive ion m/z 297 daughter experiments in conjunction with the negative ion m/z 248 daughter experiments confirm the presence of DON when present above 1.0 ppm.

MS/MS quantitative data obtained from analysis of a crude matrix, without the use of isotope dilution techniques or internal standard techniques, depend on far greater control and better understanding of the sample volatilization/ionization process than do simpler GC-MS experiments. When the instrument is in the CI mode, it is possible, if not likely, that the volatilization of the complex matrix into the CI source along with the CI reactant ions will have a significant effect on the CI reaction conditions. This effect could cause some deviation from the intensity of the molecular ion and its fragments, which are formed when the same amount of pure substance less the matrix is volatilized. Kallos et al. (11) recently discussed these matrix effects in CI-MS. They concluded that significant quantities of matrix can cause pronounced deviation from normal CI performance in the source even when "normal" CI conditions are met, and that the addition of isotopically labeled internal standards is generally the best way to compensate for matrix effects. Pure DON eluted from the probe as a distinct peak about 20 s wide at a probe temperature of about 180°C. The response of the m/z 175 daughter of the m/z 248 ion from NICI had a reasonably linear response from 2 ng to 500 ng (Table 1). When the DON was spiked into crude grain extracts, the response of the m/z 175 daughter ion from DON was spread over a considerably wider time. DON was spiked into the crude extract of mycotoxin-free corn at 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 ppm. A

Table 1. Response of m/z 175 daughter of m/z 248 from NICI MS/MS of deoxynivalenol

DON, ng	Counts	Calcd[a]
2	2700	6581
4	2979	9505
5	6776	10 968
10	11 535	18 279
20	46 085	32 901
50	106 409	76 766
100	161 615	149 876
200	256 144	296 095
500	745 138	734 759

[a] Standard curve using linear regression: counts = 3656 + (1462.2 × ng DON) correlation coefficient 0.9968.

Table 2. Response of m/z 175 daughter signal in NICI MS/MS of deoxynivalenol spiked into mycotoxin-free corn

Spike		Calcd response		
ppm	ng	ppm	ng	Response, %
0.1	25	0.036	9	36
0.2	50	0.052	13	26
0.5	125	0.15	37	30
1.0	250	0.43	108	43
2.0	500	0.68	170	34
4.0	1000	1.28	320	32

portion of the extract, equivalent to 0.025 g, was analyzed and the response of the m/z 175 daughter was measured. The abundance of m/z 175 in these spiked crude extracts was only about 40% that recorded when the DON was analyzed without any matrix (Table 2). Response of the m/z 175 daughter signal varied greatly from day to day but the data presented in Table 2 are typical for run-to-run variation. In spite of these difficulties with response, spiked mycotoxin-free wheat extracts and naturally contaminated extracts showed reasonably good comparison of MS/MS results (Table 3) with analysis for DON by EC-GC. At levels below 1 ppm, matrix effects became more important and quantitative estimates by MS/MS were not always in agreement with EC-GC data. Sometimes false negatives were observed at levels between 0.5 and 0.1 ppm due to these effects. Increasing the sample size analyzed did not improve results; however, cleanup to remove some of the unwanted matrix improved the situation. When extracts were cleaned up, using procedures similar to those reported by Scott et al. (7), MS/MS data for DON compared well with EC-GC results at concentrations as low as 0.1 ppm.

Detection of DON and zearalenone in a crude extract by MS/MS analysis below part per million levels in wheat, corn, oats, and rice is feasible. The mycotoxins were detected in crude extracts without sample cleanup. No difference

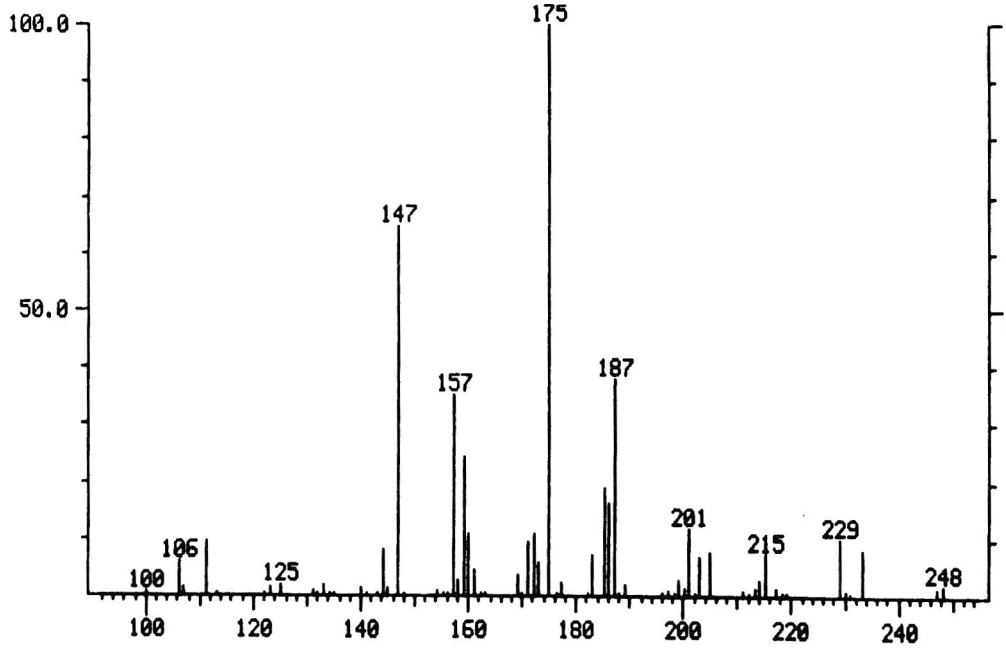

Figure 8. Argon CAD daughters of m/z 248 from DON NICI.

Table 3. Comparison of EC-GC results with MS/MS estimates of deoxynivalenol

Sample	EC-GC, ppm	MS/MS, ppm
1	2.2	1.5
2	1.0	1.0
3	0.87	1.2
4	0.89	0.45
5	0.50	0.41
6	0.22	0.38
7	0.22	0.25
8	0.23	0.16
9	0.15	0.04
10	0.11	ND
11	0.05	ND
12	0.04	0.02

in ability to detect the mycotoxins at 1.0 ppm concentrations was observed for any of the solvent extracts tested, although methanol–water (1 + 1) extracted only 50–70% of the zearalenone in the sample. Recoveries of DON and zearalenone by the other solvents was greater than 80%, with $CHCl_3$–ethanol (80 + 20) giving the best recoveries from wheat and corn (≥86%). Careful attention to instrumental parameters (particularly Q_2 pressure and probe conditions) gave reproducible daughter spectra, and comparison of response with spiked samples gave a reasonable quantitative estimate of the level of contamination in samples above 1.0 ppm. Zearalenone could be detected at 0.1 ppm, whereas DON could be detected at 1.0 ppm in the positive ion mode and 0.5 ppm in the negative ion mode. Variation of response for DON at levels below 1 ppm interfered with quantitative measurements from crude extracts of samples. A complete MS/MS experiment for both mycotoxins took less than 10 min.

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Gas Chromatographic Determination of Deoxynivalenol in Wheat

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Modifications to a published method are described for the determination of deoxynivalenol (DON) in wheat by gas chromatography with electron capture quantitation of the heptafluorobutyrate derivative. In the modified method, DON is extracted by shaking the sample with methanol-water on a wrist-action shaker, followed by filtration through rapid flow paper. One concentration step is eliminated, and a hexane wash is incorporated to remove toluene from the silica gel column. Recoveries of DON from wheat samples spiked at 0.1, 0.5, and 1.0 ppm ranged from 77.3 to 86.3% and averaged 81.5%.

Methods development for mycotoxins is an evolutionary process whereby procedures from various methods are combined or modified to improve assays or to reduce analysis time. Scott et al. (1) developed a sensitive method for the determination of deoxynivalenol (DON) in wheat by gas chromatography with electron capture (GC-EC) detection and confirmation by mass spectrometry (MS) to assess the extent of contamination in the 1980 and 1981 Canadian wheat crop. This method has a low detection limit (10 ppb), is reliable (coefficient of variation 10%), gives satisfactory recoveries (57–86%), and has been tested on a large number of naturally contaminated samples. A subsequent evaluation (2) of currently available methodology for trichothecenes concluded that GC-EC with MS confirmation is the best technique to date for determining these toxins.

In 1982, some of the hard red winter wheat harvested in eastern Nebraska and Kansas was visibly damaged (scabby) with *Fusarium* sp. and suspected of being contaminated with DON (3, 4). To expedite analysis of a large number of wheat samples, we modified the Scott procedure (1) to increase the number of samples an analyst may prepare each day. This communication describes the revised method currently used in this laboratory.

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METHOD

Reagents and Materials

(a) *Solvents.*—HPLC grade methanol, ethyl acetate, *n*-hexane, methylene chloride, toluene, acetone, acetonitrile.

(b) *Ammonium sulfate.*—30% solution.

(c) *Celite.*—Hyflo Super-Cel diatomaceous earth.

(d) *Silica gel.*—E. Merck 60, 0.063–0.200 mm particle size, containing 1% water. Activate by drying 1 h at 105°C. Add 1 mL water/100 g, seal, shake until thoroughly mixed, and store 15 h in airtight container.

(e) *Deoxynivalenol.*—Working solutions of 1 and 2 μg/mL toluene–acetonitrile (95 + 5) prepared from stock solution of 0.10 mg crystalline deoxynivalenol/mL acetonitrile. Available from Myco-Lab Co., PO Box 321, Chesterfield, MO 63170.

(f) *N-Heptafluorobutyrylimidazole (HFBI).*—1 g ampules (Pierce Chemical Co., Rockford, IL 61105).

(g) *Sodium bicarbonate.*—5% aqueous solution.

(h) *Sodium sulfate.*—Anhydrous, granular (Mallinckrodt Chemical Co., Paris, KY).

(i) *Potassium chloride.*—5% aqueous solution.

Apparatus

(a) *Shaker.*—Burrell wrist-action shaker.

(b) *Filter paper.*—Rapid flow, S&S 588 (Carl Schleicher and Schuell Co., Keene, NH).

(c) *Heating block.*—Model 2090 Temp-Block module heater (Lab-Line Instruments, Inc., Melrose Park, IL).

(d) *Vial-shaking device.*—Vortex tube mixer, or equivalent.

(e) *Chromatographic column.*—Glass, 50 × 1.3 cm od with Teflon stopcock.

(f) *Micro vials.*—2 mL, with septum caps (Regis Chemical Co., Morton Grove, IL).

(g) *Gas chromatograph.*—Bendix 2500, with 3 ft × 2 mm id glass column packed with 3% OV-101 on 100–120 mesh Gas-Chrom Q, and electron capture detector (^{63}Ni) and electron capture linearizer (Tracor Model 114556). Packard 800

chromatograph with 6 ft × 4 mm id glass column packed with 3% OV-1; ^3H detector.

(h) *Auto dispensers.*—Eppendorf with 500 and 50 μL disposable tips, or equivalent.

Sample Preparation

Grind sample (ca 1 kg) to pass 20 mesh screen and blend 15 min in Hobart planetary mixer.

Extraction

Add 250 mL methanol–water (1 + 1) to 500 mL extraction flask (Erlenmeyer with Teflon-lined screw cap) containing 50 g sample and 25 g Celite. Extract 30 min by shaking on wrist-action shaker. Filter through rapid flow paper and collect 60 mL filtrate in 500 mL beaker containing 240 mL 30% ammonium sulfate. Add 100 mL (18–19 g) Celite and stir 2 min (intermittently) with glass rod. Filter and transfer 200 mL filtrate to 500 mL separatory funnel and add 25 mL 5% aqueous potassium chloride. Shake vigorously 3 times with ethyl acetate (100 mL) and combine ethyl acetate fractions in 500 mL Erlenmeyer flask. Add 35 g sodium sulfate and swirl for 3–4 min. (If lumps form, add additional 10 g sodium sulfate.) Decant solution through funnel containing glass wool plug into 500 mL round-bottom flask. Rinse sodium sulfate with ethyl acetate (100 mL) and add ethyl acetate rinse to round-bottom flask. Evaporate to dryness under reduced pressure on rotary evaporator at ca 50°C.

Column Chromatography

Prepare silica gel column as described by Scott et al. (1) and transfer extract residue directly from round-bottom flask to column with 3 mL methylene chloride. Rinse round-bottom flask with 2 additional 3 mL washes and add rinses to column. Wash column with 30 mL acetone–toluene (5 + 95) and with 20 mL hexane and discard washes. Elute DON with 50 mL methylene chloride–methanol (95 + 5) and evaporate eluate to dryness on rotary evaporator. Transfer residue to 4 mL vial with methylene chloride, evaporate solvent under nitrogen, and seal vial with Teflon-lined screw cap. Store in refrigerator for analysis.

Derivatization

Redissolve residue from column in 4 mL toluene–acetonitrile (95 + 5) and mix on Vortex mixer. Transfer 500 μL sample solution (0.1 g) and standard DON solution (0.1 or 0.05 μg) to separate Teflon-lined screw-cap vials (15 × 45 mm). Add 50 μL HFBI reagent to each solution with disposable pipet and seal vials tightly. Agitate on Vortex mixer and heat 1 h at 60°C. Cool vial to room temperature and add 1.0 mL 5% sodium bicarbonate. Agitate vigorously on Vortex mixer for 1 min to form fine emulsion. Let phases separate (5–10 min) and carefully transfer 50 μL of upper phase to 2.0 mL vial, with septum cap, containing 950 μL *n*-hexane. Sample concentration is 100 μg sample equivalent/μL and standard concentration is 100 or 50 pg/μL.

Gas Chromatography and Quantitation

Construct a standard curve each day to determine electron capture detector response to DON standard. GC conditions were as follows: Bendix 2500, 3 ft × 2 mm column at 160°C. Inlet and ^{63}Ni detector at 210 and 280°C, respectively. Carrier gas, argon–methane (95 + 5) at 60 mL/min. Electron capture linearizer at 4 or 8× attenuation. Packard chromatograph with ^3H detector. Carrier gas, nitrogen at 50 mL/min. Inlet and detector at 210 and 220°C, respectively. Column, 180°C isothermal. Electrometer at 1×10^{-8} or 3×10^{-9} AFS. Increase column temperature to 200°C after 15–20 injections to clean columns. Record precise volume of sample and standard injected by withdrawing syringe plunger into barrel so that lower meniscus is at 1.0 μL mark. Record total volume in syringe. Inject and again withdraw plunger and measure volume remaining in syringe. Difference is volume injected. Determine concentration of DON in sample from formula:

$$\text{Deoxynivalenol, ppm} = [(\text{peak height sample}/\text{peak height std}) \times \text{pg std injected}]/\mu\text{g sample injected}$$

Results and Discussion

The revised procedure was tested on naturally contaminated and spiked wheat samples. Table 1 shows the results obtained by 4 analysts on the same naturally contaminated wheat sample. Analyst 4 used the procedure described here. The other analysts used the Scott method (1), but the extracts were prepared by shaking and filtering instead of by blending and centrifuging. The additional modifications described and used by analyst 4 reduced analysis time and gave essentially the same results obtained by the other analysts.

Percent recoveries of DON from spiked wheat are shown in Table 2. Average recovery for the 3 levels tested was 81.5%. The modified procedure was evaluated by comparing assay results

Table 1. Results of intralaboratory assay on one naturally contaminated wheat sample by modified procedure [a]

Analyst	Deoxynivalenol, ppm
1	1.49
2	1.35
3	1.33
4	1.55[b]

[a] Scott method (1) except extraction was done by shaking 30 min on wrist-action shaker.
[b] Obtained with modifications described in text.

Table 2. Recovery of deoxynivalenol from spiked wheat samples by modified procedure [a]

Sample	DON added, ppm	Rec., %	CV,[b] %
1	0.10	77.3	7.44
2	0.50	81.0	7.63
3	1.00	86.3	7.91
Av.		81.5	7.66

[a] Triplicate analyses.
[b] CV = coefficient of variation.

with those obtained on identical samples by outside laboratories which used the Scott method as published (1). A comparison of our results and results obtained by 2 different outside laboratories is presented in Table 3. The DON level in sample 2 was reported only to be greater than 14.5 ppm.

The Scott method was modified to increase the number of samples an analyst could prepare for quantitation per day. Vigorous shaking of ethyl acetate–aqueous phase containing potassium chloride unexpectedly enhanced phase separation. A hexane wash of the silica column removed residual toluene and reduced dry-down time for the DON fraction. The use of disposable pipets saved considerable time in preparing solutions for derivatization. The modifications permitted each analyst to prepare 6–8 samples per day to the step where the extract is ready for derivatization and quantitation.

Table 3. Comparison of deoxynivalenol determination in wheat by 2 methods

Sample	Deoxynivalenol, ppm [a]	
	Outside lab, Scott method (1)	NRRC[b] modified Scott method
1	0.31	0.22
2	>14.5	18.40
3	5.30	4.98
4	1.12	1.39[c]
5	6.90	7.07
6	3.20	3.65
7	7.80	5.91
8	3.40	3.74
9	10.20	10.29

[a] One determination.
[b] NRRC: Northern Regional Research Center.
[c] Five determinations.

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Enzyme-Linked Immunosorbent Assay of Ochratoxin A in Barley

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A noncompetitive, double antibody enzyme-linked immunosorbent assay for ochratoxin A using microtitration plates has been developed and applied to samples of barley. The anti-ochratoxin A antiserum, which is used at high dilution, does not cross-react significantly with ochratoxin B or ochratoxin α. Assay sensitivity for determination of the toxin in barley samples is 60 ng/kg. Minimal sample preparation is required before assay.

Ochratoxin A, a potent nephrotoxin and hepatotoxin (1), is a product of the secondary metabolism of a number of fungal species in the *Aspergillus* and *Penicillium* genera. These fungi and their products can contaminate a number of agricultural commodities, and consumption of such material has been linked with several disease states in animals and humans. Ochratoxin A is thought to be the major disease determinant in porcine nephropathy (2), and has been implicated in Endemic Balkan Nephropathy, the fatal human kidney disease found in certain areas of Bulgaria, Rumania, and Yugoslavia (3, 4).

The methods of analysis currently used for determining ochratoxin A in grain and grain products most commonly involve thin layer chromatography (5, 6) or liquid chromatography (7, 8). The availability of immunoassay procedures should present advantages to mycotoxin determination. The specificity of these assays, due to the nature of the antibody-antigen interaction, means that minimal pre-purification or sample cleanup will be necessary. Their sensitivity and precision characteristics compare favorably with alternative procedures and, in addition, immunoassays have a unique ability to routinely handle the large numbers of samples necessarily involved in monitoring programs.

Antisera to ochratoxin A have been described by 2 groups and used in radioimmunoassay systems (9, 10). This approach is limited by the absence of commercially available radio-labeled toxin of high specific activity. One of the antisera has also been used in a competitive enzyme-linked immunosorbent assay (ELISA) (11). None of the reports described the application of the assays to biological material.

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A preliminary report of these results was presented at the Vth International IUPAC Symposium on Mycotoxins and Phycotoxins, Sept. 1-3, 1982, in Vienna, Austria.

We have raised and characterized antisera to ochratoxin A and have used it to validate a noncompetitive, double antibody microtitration plate ELISA for the toxin in barley. This form of ELISA has several advantages over the competitive version. The latter requires the synthesis of a pure antigen-enzyme conjugate, preferably in a particular molar ratio. It requires that the enzyme preparation be in contact with the test sample during the assay, a situation that can present problems if the sample contains enzyme inhibitors or promoters. In addition, noncompetitive assays, which are reagent excess methods, are potentially more sensitive and have a wider working range (12). The assay described uses stable reagents, and the second, labeled antibody, is widely available commercially. The end point determination of optical density is a potentially inexpensive procedure; battery-operated portable microtitration plate readers have been described (13). The microtitration plate system is well suited to automation.

METHOD

Reagents and Apparatus

(a) *Alkaline phosphatase-labeled antisera to rabbit IgG.*—Research Products Division, Miles Laboratories Ltd, UK.

(b) *Enzyme substrate.*—*p*-Nitrophenyl phosphate (Sigma London Chemical Co., UK). Prepare fresh solution (1 mg/mL) in 0.05M carbonate-bicarbonate buffer with 0.5mM $MgCl_2$, pH 9.6, and use 0.2 mL/well.

(c) *Assay buffer.*—Dissolve anti-ochratoxin A antisera and alkaline phosphatase-labeled antisera to rabbit IgG in phosphate-buffered saline, pH 7.4, containing 0.05% (v/v) Tween-20 (PBSTween) (14). Use same buffer but including 10% (v/v) methanol to dilute ochratoxin A standards and to redissolve barley extracts.

(d) *Ochratoxin A standards.*—Aldrich Chemical Co. Ltd., UK. Quantitate spectrophotometrically and dilute as required in PBSTween containing 10% methanol (c). Prepare and characterize ochratoxin α as previously described (15).

(e) *Microtitration plates.*—Nunc Immunoplate I (Gibco Europe Ltd, UK). Wash plates with Dynawasher II (Dynatech Laboratories Ltd, UK). (This instrument will aspirate contents of the 96

wells and then fill them with wash liquid.) See text for number of cycles to use with each wash liquid. Determine microtitration plate well optical densities at 405 nm with Dynatech Microelisa Reader.

Synthesis of Ochratoxin A-Protein Conjugates

Synthesize the immunogen, ochratoxin A-bovine serum albumin, by mixed anhydride method (16). Dissolve 25 mg ochratoxin A in 2 mL dioxane, and cool to 12°C. Add 0.05 mL tri-*n*-butylamine and let stand 20 min at 12°C. Add 0.02 mL isobutylchlorocarbonate and let stand 15 min at 12°C. Add mixture to solution of 80 mg bovine serum albumin dissolved in 4.3 mL water and 2.6 mL dioxane adjusted to pH 9.5 with 1M NaOH. After mixing, bring pH of solution to 7–8 with 1M NaOH. Incubate with mixing at 4°C for 4 h. Dialyze exhaustively against water before freeze-drying. Dissolve product in 0.01M NaCl and fractionate in Sephadex G25 that has been equilibrated and eluted with the salt solution. Combine fractions containing protein, dialyze against water, and freeze-dry to give immunogen (73 mg).

Synthesize conjugate used to coat microtitration plates, ochratoxin A-keyhole limpet hemocyanin, by using a carbodiimide reagent (17). Dissolve 5 mg ochratoxin A in 0.12 mL ethanol and 3 mL 0.1M pH 7.0 phosphate buffer. Mix 51 mg keyhole limpet hemocyanin with 5 mL 0.1M NaCl. Remove undissolved material and combine with toxin solution. Add 1-ethyl-3-(dimethylaminopropyl) carbodiimide to this mixture and stir 24 h in darkness at 20°C. Purify as previously described for bovine serum albumin-ochratoxin A conjugate (17) to give the product (19 mg).

Production of Anti-Ochratoxin A Antisera

Use immunization procedure previously described (18). Store plasma at −40°C. Make up appropriate dilutions in PBSTween, and store at −20°C. For normal assay work, use anti-ochratoxin A antisera at final dilution of 1:25 000 (v/v).

Preparation of Microtitration Plates for ELISA

Form ochratoxin A solid phase by coating with ochratoxin A-keyhole limpet hemocyanin. Incubate each well with conjugate solution (0.3 mL/well of filtered 0.002 mg/mL solution in 0.05M pH 9.6 carbonate-bicarbonate buffer) for 15 h at 2°C. Wash plates once with water, and store under desiccation. Before use, wash once with PBSTween.

ELISA Protocol

Add 0.1 mL samples or standards in triplicate as required and 0.1 mL anti-ochratoxin A antisera to appropriate wells of coated microtitration plates. Incubate for either 3 h at 35°C or 15 h at 2°C. Wash plates 5 times with PBSTween. Add alkaline phosphatase-labeled antisera to rabbit IgG (diluted 1:4000 (v/v) in PBSTween; use 0.2 mL/well). Incubate 3 h at 35°C. Add 0.2 mL enzyme substrate/well. Incubate 1 h at 35°C, and record well optical densities. Determine ochratoxin A content of samples by reference to standard curves.

Determine cross-reactions by modification of standard definition (19). Take ratio (as percentage) of mass of compound of interest given by 50% of maximum absorbance to mass of ochratoxin A defined by same value.

Sample Preparation for ELISA

Weigh finely ground barley samples (5 or 10 g as required) into 250 mL round-bottom flask, and add 6 mL 0.1M phosphoric acid and 60 mL chloroform. Shake 30 min on wrist-action shaker. Let stand 10 min. Remove 10 mL aliquot liquid phase. Evaporate to dryness with rotary evaporator and re-dissolve in PBSTween including 10% methanol (≥1 mL as required to give appropriate ochratoxin A concentrations). Take 0.1 mL aliquots for ELISA.

Spike barley by adding ochratoxin A in chloroform to ground barley. Let solvent evaporate and proceed with extraction.

Results and Discussion

The ochratoxin A-keyhole limpet hemocyanin conjugate that was synthesized was used to coat the microtitration plates to form the solid phase aspect of the ELISA. The coated plates have shown no evidence of deterioration on storage; it has been reported that coated plates are stable for a least 1 year if stored dry (14).

The other synthesized conjugate, ochratoxin A-bovine serum albumin immunogen, had a hapten-protein ratio of 22:1 determined spectrophotometrically. All rabbits responded well to immunization, and one particular breed was used throughout the work described. The final antiserum dilution used was 1:25 000 (v/v).

High dilution is important for reasons of economy and continuity and to eliminate nonspecific factors. The antiserum enabled production of a sensitive standard curve for ochra-

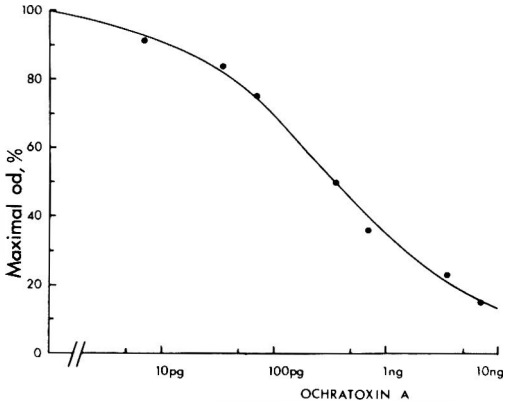
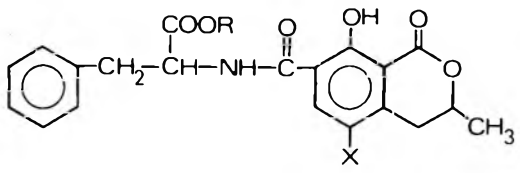


Figure 1. Standard curve for ochratoxin A determination by ELISA. Nonspecific binding (no ochratoxin A, no antibody) was less than 5%. Results are means of triplicate determinations.



	R	X
Ochratoxin A	H	Cl
Ochratoxin B	H	H

Figure 2. Structures of ochratoxins A and B.

Table 1. Recovery of ochratoxin A added to barley

Added, μg/kg	Rec., % (CV, %, n = 5)
0.48	96.7 (8.8)
1.92	97.9 (12.4)
19.20	95.8 (6.5)
48.00	95.0 (6.3)

toxin A determination under the conditions described (Figure 1). The least detectable amount (that significantly different from zero) was 10 pg ochratoxin A/well (P <0.001; n = 10).

Cross-reactions of compounds of interest tested in the ELISA system are as follows: ochratoxin A, 100; ochratoxin B, 0.5; ochratoxin α, 2.4; coumarin, <0.00005; 4-hydroxycoumarin, <0.00005; and phenylalanine, <0.00005%.

Figure 2 shows the structures of ochratoxins A and B. Ochratoxin α is a mammalian metabolite of ochratoxin A (20), resulting from the loss after hydrolysis of the phenylalanine moiety. The antiserum is very specific for ochratoxin A. Absence of the chlorine atom in ochratoxin B reduces recognition considerably, to 0.5%. The lack of antibody binding to phenylalanine and the coumarin compounds and the much reduced binding to ochratoxin α (only 2.4%) suggest that at the high serum dilution used in the assay the predominant binding effects are for the total ochratoxin structure only.

In applying the ELISA to the determination of ochratoxin A in barley, we found that sample preparation could be kept to a minimum. Thus, extraction of the toxin from ground barley yielded a liquid phase that could be assayed directly after solvent evaporation and re-dissolution in the appropriate buffer. The specificity of the antiserum is therefore utilized fully, and the resultant simple work-up clearly has considerable advantages over methods of analysis requiring lengthier procedures.

The ELISA is potentially a very sensitive method. Under the conditions described, the limit of detection would be equivalent to 60 ng toxin/kg barley. Naturally contaminated barley samples showed linearity of response for ochratoxin A content against dilution of barley extract assayed. Portions of the chloroform extract of the barley (10 mL) were taken to dryness and re-dissolved in assay buffer. Different volumes (0.02, 0.04, 0.06, 0.08, and 0.1 mL) were then transferred to the microtitration plate for assay, with the total incubation volume after addition of antibody made up to 0.2 mL with buffer. Volume of extract assayed was plotted against ochratoxin A content. Samples containing 10 and 129 ng/g ochratoxin A (as determined by ELISA) gave linear dilution plots (r = 0.995 and 0.984, respectively) and intercepts of −0.0095 and −0.0087 mL, respectively. These results indicate the absence of significant nonspecific factors in the immunological procedure.

Currently accepted alternative methods of analysis for ochratoxin A in barley specify extensive sample cleanup procedures. The high specificity of our antiserum preparation means that we have been able to reduce sample preparation to the simple solubilization step, which is generally applied as the initial procedure in alternative methods. Recovery of toxin spike would not, therefore, be expected to be a problem, and this was the case. Table 1 shows the results of recovery experiments, which have been carried out on both naturally contaminated

material and on samples in which no endogenous ochratoxin A could be found.

The intra- (within-) assay coefficient of variation (CV) for the method was determined from replicate analyses of a single barley extract on a single plate. The inter- (between-) assay CV value was determined from replicate analyses of separate barley extracts, each assayed on separate plates. Barley samples containing toxin at 3.22 and 29.9 ng/g gave intra-assay CV values of 2.5 and 3.1%, and inter-assay CV values of 5.5 and 7.0%, respectively (n = 10 in each case).

The properties of the antiserum and the noncompetitive sandwich ELISA standard curve described compare favorably with the previous reports of anti-ochratoxin A antiserum preparation. The first, a radioimmunoassay (9), had a standard curve sensitivity of 20 ng. No cross-reaction data were presented. The second (11) used antiserum diluted 1:200 (v/v) in a direct ELISA system. The specificity of the antiserum was not as good with respect to ochratoxin B, having a cross-reaction of 14% compared with 0.5%, but was slightly better with respect to ochratoxin α (1.3% compared with 2.4%).

Application of the ELISA assay to barley illustrates the potential of the immunological analytical approach. Samples can be prepared simply and quickly, and the subsequent assay is sensitive and specific. Work is now being carried out to assess ELISA performance in the routine determination of ochratoxin A in barley.

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Analysis for *Fusarium* Toxins in Various Samples Implicated in Biological Warfare in Southeast Asia

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Samples of leaves, water, cereal grains, soil, and yellow powder as well as blood, urine, and body tissues from chemical warfare victims were analyzed for *Fusarium* toxins by using gas chromatography and mass spectrometry. The leaves, water, and yellow powder samples contained various combinations of T-2 toxin, diacetoxyscirpenol, deoxynivalenol, nivalenol, and zearalenone in concentrations ranging from trace (1.0 ppb) amounts to 143 ppm. These trichothecenes do not occur naturally on the substrates described and were correlated with the so-called "yellow rain" chemical attacks against Hmong people in Southeast Asia. Analysis of leaves, soil, water, and cereals collected in areas adjacent to but apart from the area where chemical attacks had been staged did not contain any *Fusarium* toxins. Moreover, T-2 and HT-2 toxins were found in human blood, urine, and body tissues (heart, esophagus, kidney, lung, and large intestine) of alleged victims. In addition, diacetoxyscirpenol was found in the kidney of one person who had died.

The trichothecene metabolites produced by various species of *Fusarium* are secondary natural products with inherent, unique biological activities. The best known of the trichothecenes is T-2 toxin [4β,15-diacetoxy-8α-(3-methylbutyryloxy)-3α-hydroxy-12,13-epoxytrichothec-9-ene]. T-2, as well as diacetoxyscirpenol (DAS), deoxynivalenol (DON), and nivalenol (NIV), have been found occurring naturally. Of the 4 toxins, DON is most frequently encountered in nature, particularly in maize and wheat, and is often associated with zearalenone. The structures of these mycotoxins are shown in Figure 1.

Most of the trichothecenes are potent skin irritants and inflammatory agents; their toxicity has been established in rats (1–3), in rainbow trout (2), in dairy cattle (4–6), in swine (7–9), in chickens (10,11), in cats (12,13), and in guinea pigs (14).

The trichothecenes have been implicated in a disease described in the Soviet Union before 1900 and called alimentary toxic aleukia (ATA). The disease had a serious impact on the Soviet population. In one episode, 10% of the population in the Orenburg district was affected by eating bread and cereals from overwintered, *Fusarium*-infected grain. Thousands of fatalities were recorded; in one episode, an entire peasant village was almost annihilated. Joffe (15) described the effect on humans as follows: In the first stage, the patient feels a burning sensation in the mouth, esophagus, and stomach, followed by vomiting, diarrhea, and abdominal pain; in the second, there is a marked decrease in leukocytes, agranulopenia, and lymphocytosis; in the third stage, petechial hemorrhages develop on various upper parts of the body (chest, arms, thighs, face) and necrotic areas develop in the throat; the fourth stage is one of convalesence or death, and, if the patient survives, it may take up to 2 months for the blood-forming capacity of the marrow to return to normal. The disease is also characterized by necrotic angina, sepsis, exhaustion of the bone marrow, fever, and bleeding from the nose, throat, and gums.

Natural intoxication of humans also took place in northern China on the border of the Soviet Union, where victims complained of dizziness, nausea, fever, and diarrhea (16). The patients became ill after ingesting bread made from cereals infected with *Fusarium*, which they appropriately called "drunken bread."

The toxin causing ATA was described as being a steroid and was called poaefusarin (17). Mirocha and Pathre (18) analyzed a sample of poaefusarin and found that it consisted of T-2 toxin, neosolaniol, T-2 tetraol, and zearalenone; no traces of a steroid were present. The authors concluded that the major toxic component was T-2 toxin.

Since 1975, the U.S. government has received consistent reports detailing chemical attacks in Southeast Asia (Laos and Kampuchea), which, for the most part, were perpetrated against the civilian Hmong people. Some of these reports described the use of lethal agents which produced symptoms in humans that could not be

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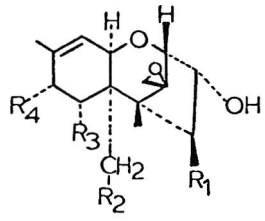

KEY	R_1	R_2	R_3	R_4
T-2 toxin	CH_3COO-	CH_3COO-	H	$(CH_3)_2CHCH_2COO$-
Diacetoxyscirpenol	CH_3COO-	CH_3COO-	H	H
Deoxynivalenol	H_2	OH	OH	O
Nivalenol	OH	OH	OH	O

Figure 1. Structure of trichothecenes commonly encountered and suspected of causing mycotoxicoses.

correlated with those produced by known or traditionally recognized chemical warfare agents or combinations. Symptoms included prolonged vomiting, diarrhea, hemorrhage, breathing difficulty, itching and skin irritation, nausea, blurred vision, headache, fatigue, dizziness, vertigo, and death. When these symptoms were compared with those induced by known chemical warfare agents, correlation was poor. These symptoms best fit those caused by the trichothecenes, never before suspected of being used as chemical warfare agents.

In the fall of 1980, the trichothecenes were added to the list of chemical agents suspected to have been used in Southeast Asia and Afghanistan. In March 1981, the U.S. Army's Chemical Systems Laboratory (CSL) reported the presence of an unusual compound ($C_{15}H_{24}$) in the vapor analyses from several clothing and tissue samples taken from the victim of a chemical attack. The compound closely resembled the structure of a trichothecene and hence sparked the request that all future samples be analyzed for the presence of trichothecene mycotoxins. Since then, Rosen and Rosen (19) independently obtained yellow powder samples from ABC News and confirmed our original observations, i.e., presence of T-2, diacetoxyscirpenol, and zearalenone.

Experimental

Extraction and Cleanup Procedures

Samples for analysis were obtained from the U.S. State Department. The leaf and stem samples (FS698A and B) were extracted with acetonitrile twice and concentrated on a steam bath. Samples were cleaned up by a ferric-gel procedure: $FeCl_3.6H_2O$ was added to water (15% final concentration) and adjusted to pH 4.6 with 0.1N NaOH. The sample in acetonitrile was added to the gel, stirred, and allowed to equilibrate 3–4 min. The ferric-gel mixture was filtered through fluted Whatman No. 4 paper, and the effluent was partially concentrated on a steam bath. The aqueous solution was cooled, transferred to a separatory funnel, and partitioned 3 times with methylene chloride. The methylene chloride extracts were dried with anhydrous sodium sulfate and then evaporated to dryness. The concentrate was transferred quantitatively with acetone to a ½ dram vial and prepared for analysis by combination gas chromatography-mass spectroscopy (GS-MS).

Sample FS704C (scraping from rock) was extracted with acetonitrile, filtered through a sintered glass filter, concentrated, and transferred to ½ dram vials with acetone.

Sample FS704A (water and unidentified tissue) was evaporated under nitrogen, extracted with acetonitrile, concentrated, and prepared for analysis by GC-MS as described above. The yellow powder (FS704B) was extracted with acetonitrile, filtered, and partitioned with petroleum ether (bp 60–70°C); both phases were saved and concentrated. The petroleum ether layer was spotted on a thin layer chromatographic (TLC) plate (silica gel 60) and developed in toluene–ethyl acetate (75 + 25). An identical petroleum ether fraction was obtained from a laboratory culture of *Fusarium roseum* for comparison.

Samples FS709A-I consisted of water, black fine soil, leaves, and kernels of rice and corn. The leaf samples (FS709F-I) were extracted with acetonitrile, defatted with petroleum ether, concentrated, diluted in 5% aqueous methanol, and added to an XAD-2 column (10 × 1 cm bed volume). The column was rinsed with water and eluted with methanol–water (90 + 10). The eluate was saved for GC-MS analysis. The water sample (FS709A) was extracted with ethyl acetate, concentrated, and saved for GC-MS analysis. The extracted water was evaporated and the residue was extracted with acetonitrile, concentrated, and saved for analysis. The soil samples (FS709B and C) were extracted with 95% methanol (3 times), filtered through Whatman No. 1 paper, concentrated on a steam bath, and transferred to a ½ dram vial with acetone. The rice and corn samples (FS709D and E) were extracted with acetonitrile and purified by the ferric gel procedure as described.

Human blood samples (FS712A-Q) were precipitated with acetone (2 times the sample volume). The supernatant solution (acetone) was evaporated with nitrogen, dissolved in metha-

nol–water (1 + 19 v/v), and eluted through an XAD-2 column as described before.

Human urine samples were passed through an XAD-2 column and the methanol-water (90 + 10) eluate was collected. If necessary, samples were further cleaned up by passing them through a Sep-Pak C18 cartridge. Human tissue samples were received fixed in formalin. (We had determined before analysis that formaldehyde does not degrade the T-2 molecule.) The tissues were homogenized in 100 mL acetonitrile and extracted with this solvent 3 times. The combined filtrate was evaporated almost to dryness and redissolved in 10 mL methanol. To this solution, 50 mL water was added, the resulting volume was concentrated to 20 mL, and the concentrate was added to an XAD-2 column. The column was washed with 100 mL water and the trichothecenes were eluted with methanol-water (90 + 10). The eluate was concentrated to dryness and the corresponding trifluoroacetate esters of the trichothecenes were prepared by reacting with trifluoroacetic acid anhydride or methylene-bis-trifluoroacetamide (MBTFA, Pierce Chemical Co.).

Gas Chromatography – Mass Spectrometry

Final resolution and quantitation of all samples was done on a Hewlett Packard 5985B combination gas chromatograph-mass spectrometer complete with an updated computerized data analysis system (Answer software, Hewlett Packard). Analyses were done in either electron impact or positive chemical ionization mode in methane or ammonia. Columns used for resolution of the mixtures were either 3% OV-17 packed on 80–120 mesh Chromosorb B or 30 m × 0.25 mm DB5 capillary with covalently bonded phase inserted directly into the spectrometer source.

Results

Because of extreme difficulties involved in collecting samples from combat zones, all samples obtained were minimal in quantity and hence the latitude in the type of analysis was limited; often, there was only enough sample for one attempt at analysis. As an example, our laboratory normally uses 25–50 g sample for extraction and, after isolation and cleanup procedures, 1–10 g equivalents are injected into the analytical instrument. With 0.2 g samples of leaves, we were able to inject, at maximum, a 20 mg equivalent. Without the analytical power of the mass spectrometer, interfaced to a gas chromatograph, these analyses would not have been possible.

At the time the first samples were received, we were not aware of the international significance of the analyses and treated the samples routinely as we do with hundreds of other agricultural samples. We were not informed which of the samples were principals and which were controls or if controls were submitted. This procedure was continued for all subsequent analyses reported in this paper.

The first leaf samples that we received (July 1981) appeared to be fragments of leaves and petioles (FS698A and B), and a water sample (Table 1). They were collected within 24 h of an attack in an area (TV3391) located just south of Phnom Mak Hoeun. The vegetation sample (0.2 g sample size) contained 109 ppm nivalenol, 59 ppm deoxynivalenol, and 3.17 ppm T-2 toxin. T-2 toxin had been added to another leaf sample (FS698B) to provide an internal positive control;

Table 1. Analysis by GC-MS of *Fusarium* toxins from various samples originating in Southeast Asia

Sample	No.	*Fusarium* toxins, ppm[a]				
		T-2	DON	NIV	DAS	Zearalenone
Dried leaf[b]	FS698A = 1 (0.2 g)	3.17	59	109.0	–	–
Dried leaf[b]	FS698B = 2 (0.2 g)	35.7	0	21.7	–	–
Water (10 mL) with unidentified tissue[c]	FS704A = D8 (33 mg)	0	66[a]	0	296 ppb[a]	0
Yellow-green powder[c]	FS704B = E168	143	0	0	27	40[d]
Unidentified fragment[e] (1 mm)	FS704C = F1-2 (1 mg)	0	0	0	10 ng/vial (~10 ppm)	–

[a] 0 = Not detected; (–) = not looked for. Concentrations of DON and DAS based on 33 mg (dry weight) of tissue are 66 ppm and 296 ppb respectively, and 0.2 ppm and 1.0 ppb on the basis of 10 mL water.

[b] Samples received July 1981.

[c] Samples received October 10, 1981 (another subsample of powder was obtained October 23, 1981).

[d] Full mass spectrum of zearalenone was obtained.

[e] Scraping from rock.

35.7 ppm T-2 and 21.7 ppm nivalenol were found. We did not attempt to analyze for diacetoxyscirpenol and zearalenone (a phytoestrogen produced by *Fusarium* spp.) because of the limited sample size. The selected ion monitoring profile and full mass spectrum of the trifluoracetate ester of T-2 toxin found in the vegetation is shown in Figure 2. Components found

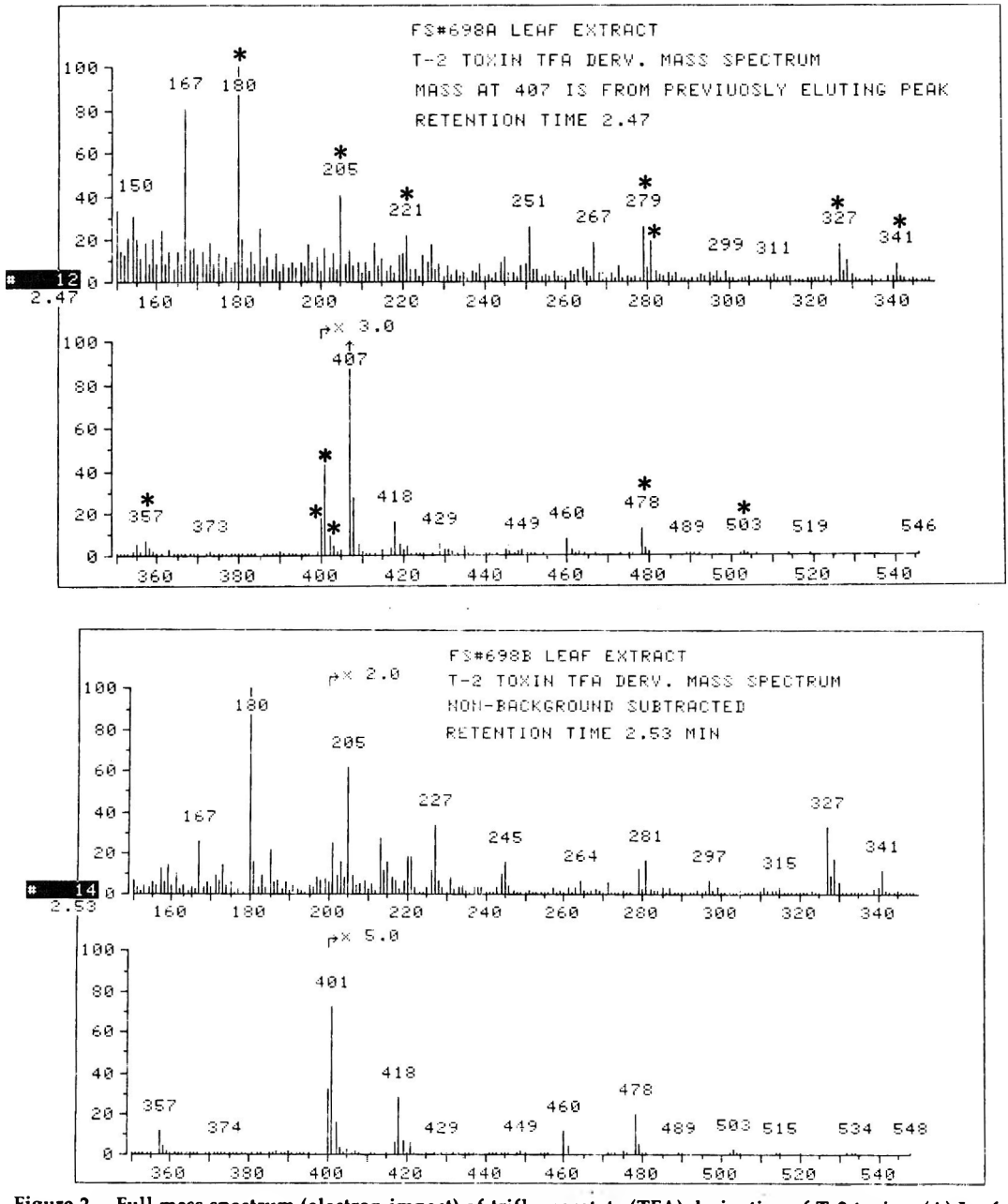

Figure 2. Full mass spectrum (electron impact) of trifluoracetate (TFA) derivative of T-2 toxin. (A) Leaf extract analysis of stem fragment FS-698A. Significant masses marked with asterisk. Note cluster at m/z 400, 401, and 402, used as diagnostic ions. (B) Extract of leaf ammended with T-2 toxin. Electron impact mass spectrum is identical to authentic T-2 and is shown as comparative T-2 standard spectrum. Normally, molecular ion (M^+ = 562) does not appear in EI spectrum from quadrupole instrument. The chromatographic retention times of A and B are identical to authentic T-2 toxin.

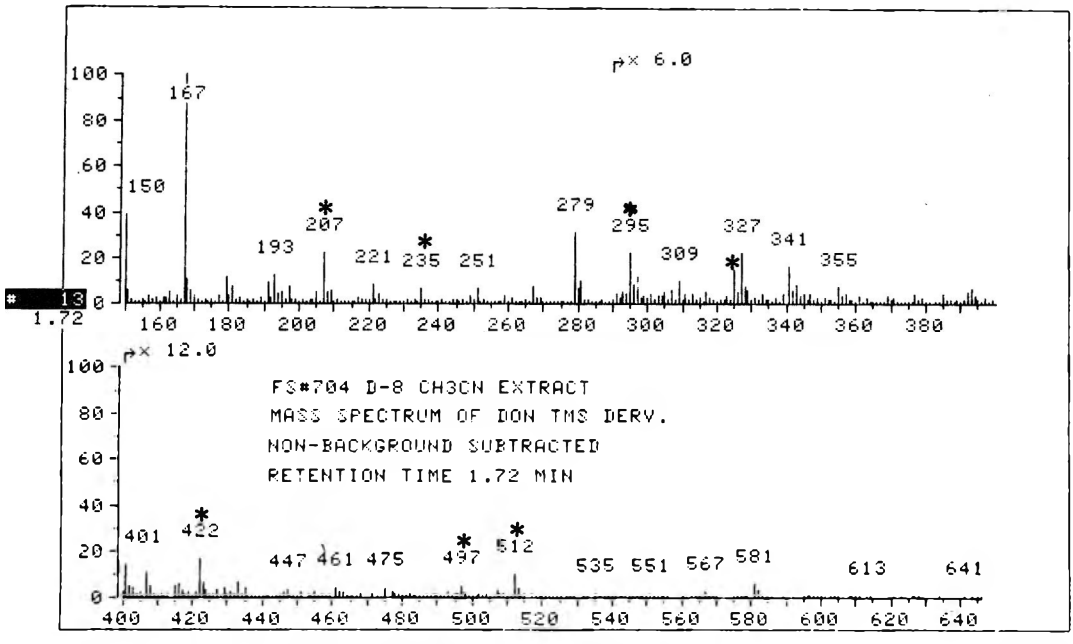

Figure 3. Full electron impact mass spectrum of trimethylsilyl ether derivative of deoxynivalenol (DON) found in water sample FS-704 (D-8). Significant masses are marked with asterisk. Molecular ion (M^+) is 512, $M^+ - 15$) = 497, ($M^+ - 90$) = 422. Retention time is identical to authentic TMS-DON; m/z 581 is not part of DON spectrum.

in the leaf samples were resolved on a 1 m × 4 mm glass column of 3% OV-101 on Chromosorb, using a single jet separator.

A 10 mL sample of water collected from the same site as the leaf samples was submitted for analysis on October 10, 1981. Sample FS704A(D8) was composed of water with unidentified tissue (33 mg) floating in it. It was impossible for us to determine the nature of the tissue and at the same time there was some confusion as to whether we should analyze the water, the tissue, or both; both were analyzed. Sixty-six ppm deoxynivalenol (DON) and 296 ppb diacetoxyscirpenol (DAS) was contained in the tissue sample. A full mass spectrum of the trimethylsilyl (TMS) ether of DON is shown in Figure 3. The sample did not contain enough DAS for a full mass spectrum.

Less than 1.0 mg was available from a sample (FS704C) that was scraped off a rock (identification of rock scraping unknown) located in a chemical attack site at Ban Thong Hak (TF9177). The attack occurred at 2:00 PM on April 2, 1981. About 10 ng diacetoxyscirpenol was found in this sample; T-2, DON and nivalenol could not be detected.

On September 20, 1981, samples of vegetation, soil, and cereals which were to serve as controls were collected from an area around TV3391 that had not been subjected to any reported chemical attacks. The samples were collected by U.S. personnel under instructions to reproduce the sampling conditions of the original samples as closely as possible. The controls consisted of 4 leaf samples, one rice and one corn sample, 2 soil samples, and one water sample. These were analyzed for T-2, DAS, DON, and nivalenol; all were negative (Table 2).

A vial containing yellow-green powder-like material (140 mg) was received on October 10, 1981. It had originated from a "yellow rain" attack on March 13, 1981, in the village of Muong Cha (TF9797) in the Phou Bia region of Laos. The agent was sprayed from a twin-engine propeller aircraft and was described as falling "like an insect spray" and sounded like drizzling rain. The sprayed substance was sticky at first but soon dried to a powder. Symptoms described by victims included nausea, vomiting, and diarrhea. This sample was scraped from the surface of a rock, carried into Thailand, and turned over to U.S. Embassy personnel.

The extract of the above sample (140 mg) was accidentally spilled in our laboratory and a subsample (50 mg) was obtained on October 20, 1981. The extract of the yellow powder sample

Table 2. Analysis of leaf, soil, water, corn, and rice collected as background control samples originating in Southeast Asia [a]

Sample	Amount, g	Identity No.	T-2	DAS	NIV	DON
Water	50	709A (M26-81A_1)	0	0	0	0
Soil	0.6	709B (M26-81B_1)	0	0	0	0
Soil	0.5	709C (M26-81C_1)	0	0	0	0
Corn	25.0	709D (M26-81D_1)	0	0	0	0
Rice	25.0	709E (M26-81E_1)	0	0	0	0
Leaf	1.4	709F (M26-81F_1)	0	0	0	0
Leaf	0.5	709G (M26-81G_1)	0	0	0	0
Leaf	1.3	709H (M26-81H_1)	0	0	0	0
Leaf	1.3	709I (M26-81I_1)	0	0	0	0

[a] Leaf samples were moist in plastic bag when received. All other samples except water were dry. Samples were obtained February 1, 1982.

was resolved on a 15 m covalently-bonded phase (CPDB5) capillary column and contained 143 ppm T-2 toxin and 27 ppm DAS. The full chromatographic profile (total ion current) is shown in Figure 4, and the full mass spectra of the trifluoroacetate esters of T-2 and DAS are shown in Figures 5A and B. The yellow powder also contained 3 yellow pigments (partitioned into petroleum ether) which were resolved on silica gel G by thin layer chromatography. These yellow pigments appeared to be identical to the pigments found in cultures of *Fusarium roseum* growing in our laboratory. Zearalenone (40 ppm) was also found in the sample and its mass spectrum is presented in Figure 6.

Human blood samples were drawn on October 7, 1981, from victims exposed to a chemical attack near Takong on September 19, 1981. The heparinized whole blood samples packed in ice were received in our laboratory for analysis on November 9, 1981. Samples were extracted as described and analyzed for products of T-2 metabolism: T-2, HT-2, TMR-1, and T-2 tetraol. One of the samples [FS-712F(B_6)] was used completely in developing our method of extraction, and Sample FS-712N(A_{14}) was too small a volume

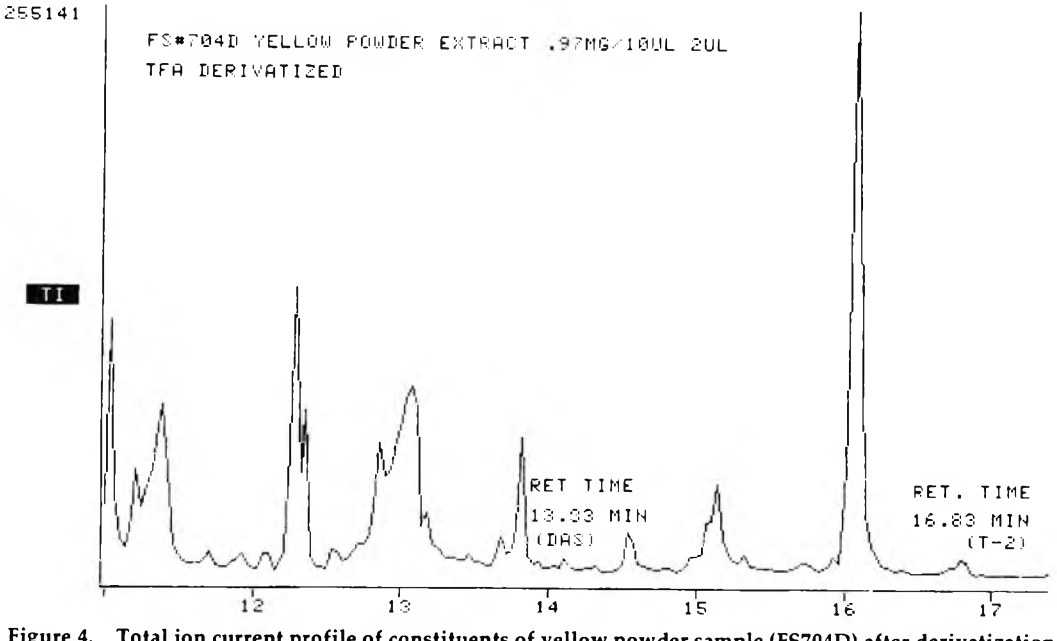

Figure 4. Total ion current profile of constituents of yellow powder sample (FS704D) after derivatization with trifluoroacetic acid anhydride and resolution on 15 m capillary column. Individual peaks are all normalized to largest component at retention time 16.1.

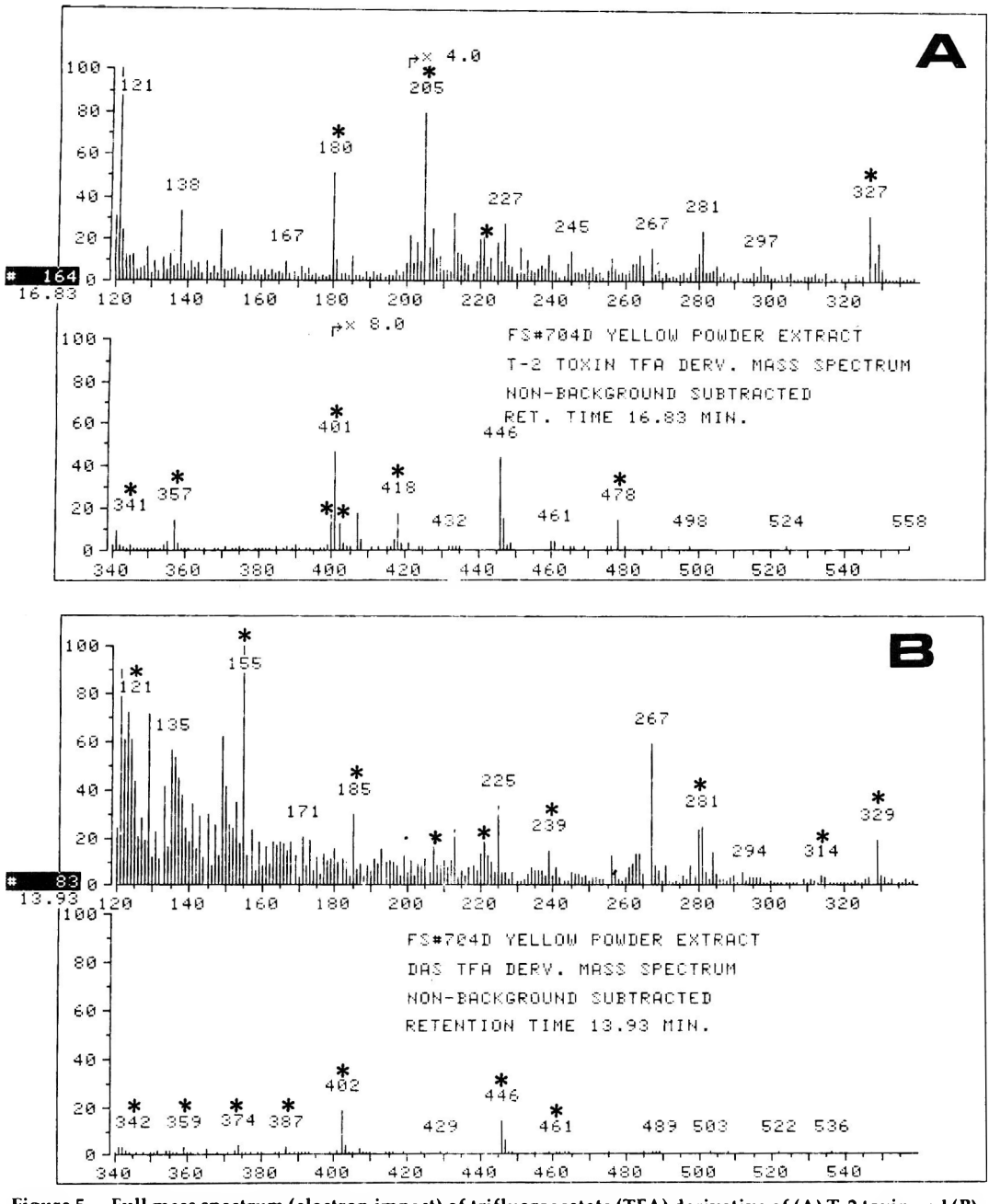

Figure 5. Full mass spectrum (electron impact) of trifluoroacetate (TFA) derivative of (A) T-2 toxin and (B) diacetoxyscirpenol (DAS) found in yellow powder sample FS-704B. Significant masses are marked with asterisk. Chromatographic retention times of T-2 and DAS were identical to authentic standards.

to analyze. All other blood samples were negative except for FS-712C(B_3) and FS-712D(B_4). The latter 2 samples were resolved by capillary columns and analyzed by selected ion monitoring (SIM). HT-2 was tentatively identified in the samples by SIM but, because of the limited sample size and quantities detected, confirmation via a full mass spectrum could not be obtained (Table 3).

Additional whole blood, serum, and urine

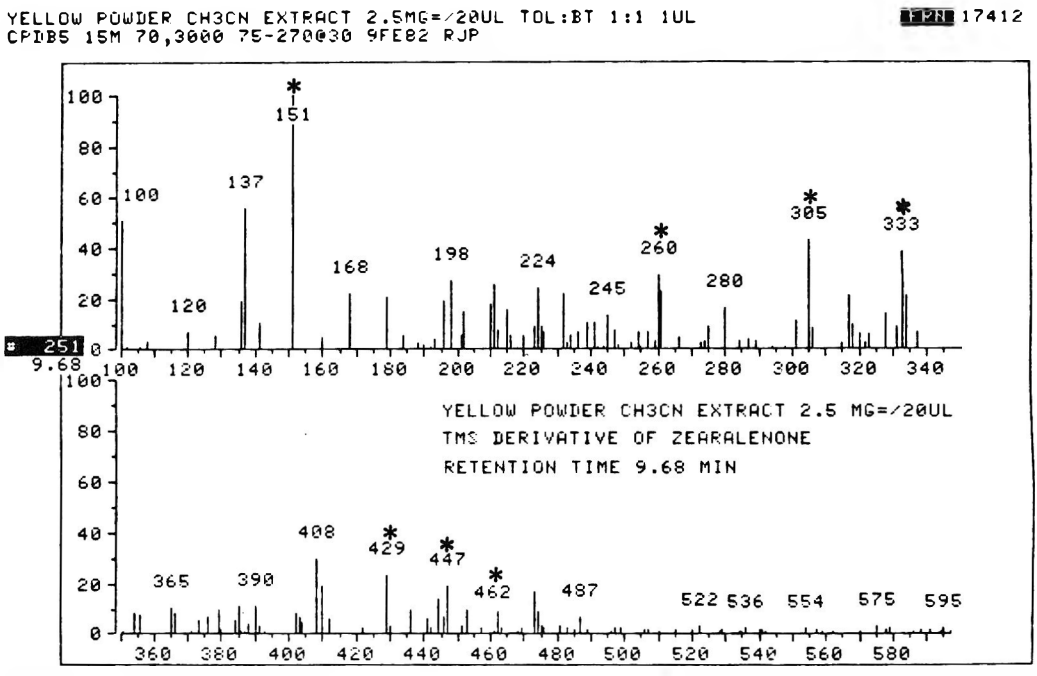

Figure 6. Full mass spectrum of trimethylsilyl ether of zearalenone found in yellow powder sample (FS704B = E168) collected from attack area. The M^+ (462) and m/z at 151, 260, 305, 333, 429, and 447 are diagnostic for zearalenone. Background was not subtracted from this spectrum. Chromatographic retention time on 15 m capillary column was identical to authentic zearalenone.

samples were obtained from victims of a chemical attack that occurred on February 13, 1982, at Tuol Chrey in Kampuchea. The victims (FS726B and FS726F) sought medical assistance in the Phum Tmey camp. A group of Western physicians collected blood and urine samples from the victims less than 24 h after exposure. The 2 victims reported severe eye irritation, prolonged and repeated episodes of vomiting, difficulty in breathing, trembling, and severe diarrhea.

Table 3. Analysis of human blood samples from victims exposed to biological warfare chemicals in Southeast Asia [a]

Sample No.		Trichothecenes			
		T-2	HT-2	TMR-1	T-2-4ol
FS712A-B	(B_1)	0	0	0	0
FS712C	(B_3)	0	trace	0	0
FS712D	(B_4)	0	trace	0	0
FS712E-Q	(B_5-B_{17})	0	0	0	0

[a] Samples A_{14} and B_6 were not tested. The quantity of blood in Samples FS712A-M was approximately 15 mL; Samples N-Q, approximately 2 mL. A tentative identification of HT-2 was made based on selected ions. Full mass spectra could not be obtained because of the limited amount of material available. Samples were received November 9, 1981 and were drawn October 7, 1981, 18 days after exposure to the chemical attack.

The blood and urine samples were resolved on a 30 m DB5 capillary column and analyzed by positive chemical ionization with methane ionization gas. Blood drawn from FS726B showed a level of 18 ppb T-2 toxin and 22 ppb HT-2 toxin while blood from FS726F contained 11 ppb T-2 and 10 ppb HT-2. The urine sample collected from FS726D 48 h after exposure contained a trace quantity of T-2 and 18 ppb HT-2. Additional blood samples were collected by a physician with the International Rescue Committee on March 3, 1982, 18 days after the attack. Samples of blood from 2 (FS726H and FS726L) of the 6 individuals contained 7 and 3 ppb, respectively, of T-2 toxin. Analysis of blood samples from control individuals of similar age and background who had not been subjected to chemical attack were negative for the trichothecene toxins. Consult Table 4 for the original data and Figure 7 for the positive chemical ionization spectrum of T-2 toxin found in the blood of victim FS726B.

Additional blood and urine samples were drawn from victims exposed to aerial delivery of "yellow rain" toxins in Laos and Kampuchea and

Table 4. Analysis of whole blood samples and urine from human victims of chemical warfare in Southeast Asia [a]

Sample identification	T-2 toxin, ppb	HT-2, ppb
FS726A(A1) urine	ND	ND
FS726D(B1) urine [b]	trace	18
FS726B(A2) blood [b]	18	22
FS726F(B3) serum [b]	11	10
FS726H(R2) blood [c]	7	ND
FS726L(R6) blood [c]	3	ND
FS726C-E-G-I-J-K-M-N-O-P-Q-R blood [c]	ND	ND

[a] ND = Not detected. Quantitation based on HT-2. Analyses made by positive chemical ionization. Samples received March 21, 1982.

[b] See Figure 7 for chemical ionization data for T-2 and HT-2; samples were drawn 24 h after attack. Victims FS726B and FS726F.

[c] Blood samples were drawn 18 days after initial exposure. Victims FS726H and FS726L.

Table 5. Analysis of whole blood and urine for trichothecene toxins from human victims of chemical warfare [a]

Sample identification	T-2, ppb	HT-2, ppb	DAS, ppb
FS736-7 (blood) M-33-82-N7	–	24.3	–
FS736-12 (blood) M-33-82-N13	25.9	–	–
FS736-13 (blood) M-33-82-A2 [b]	13.5	–	–
FS736-20 (blood) M-54-82-J2 [c]	110.4	296.1	–
FS736-21 (blood) M-54-82-K1 [d]	100.7	8.2	–
FS736-22 (blood) M-54-82-J3 [e]	46.2	–	–
FS736-23 (blood) M-54-82-L1 [f]	19.4	–	–
FS736-24 (blood) M-54-82-L2 [f]	2.7	2.0	–
FS736-28 (blood) M-33-82-4	1.4	–	–
FS736-28 (blood) M-33-82-4	1.4	–	–
FS736-30 (blood) M-33-82-X6	72.5	22.0	–
FS736-31 (blood) M-33-82-X7	7.7	97.3	–
FS736-32 (blood) M-33-82-X8	16.1	14.4	–
FS736-33 (blood) M-33-82-X9	47.6	28.4	–
FS736-34 (blood) M-33-82-X11	58.8	–	5.0
FS736-40 (blood) M-54-82-02 [g]	14.5	19.2	–
FS736-42 (blood) M-24-82-BR1 [h]	6.9	–	–
FS736-43 (blood) M-24-82-BR2 [i]	–	8.1	–
FS736-44 (blood) M-55-82-K2 [d]	32.9	34.0	–
FS736-H (urine) M2-82-U1 [h]	5.0	1.8	–
FS736-I (urine) M2-82-U2 [i]	4.0	1.3	–
FS736-J (urine) M2-82-U3 [i]	22.0	7.4	–
FS736-1-6; 8-11; 14-19; 25-27; 29; 35-38; 41 (25 blood samples)	–	–	–

[a] (–) = not detected.

[b] Male Hmong victim twice exposed to chemical agent in Laos.

[c] Laos Victim.

[d] Laos Victim.

[e] Laos Victim.

[f] Hmong refugee Laos.

[g] Hmong refugee 25 yr-old who died after exposure (Laos).

[h] Kampuchea Victim.

[i] Kampuchea Victim.

delivered to our laboratory for analysis (Table 5). Blood was obtained from a Hmong couple exposed to what was described as a yellowish and greenish chemical delivered, respectively, on November 11, 1981, and January 4, 1982. Blood samples were drawn on March 21, 1982, and analyzed for DAS, T-2, and HT-2. The blood of the male (FS736-13) contained 13.5 ppb T-2, whereas that of the female was negative (FS736-14). Three blood samples were drawn by a U.S. physician on April 17, 1982, from three Hmong refugees: an 8-year-old boy, a 6-year-old boy, and a young girl. They were exposed in Laos late in March 1982 to an agent described as yellow to reddish brown and their blood was sampled on April 17, 1982. The 8-year-old boy had been severely ill with bloody diarrhea and coughing of blood, but his analyses were negative. The girl was suffering from bloody diarrhea and abdominal pain. The 6-year-old boy's blood (FS736-20) contained T-2 and HT-2 and the girl's blood (FS736-22) contained T-2.

Post-mortem blood samples were obtained from a 25-year-old male Hmong refugee who had been admitted to a hospital in Ban Vinai, Thailand. He indicated exposure at some undetermined time in Laos and blood was drawn on April 17, 1982. Just before death, the victim suffered a massive gastrointestinal hemorrhage. Both T-2 and HT-2 were found in the blood (FS736-40).

A Hmong refugee in Thailand suffered from a chemical attack that occurred in Phou Bia, Laos, on March 25, 1982. Her blood was sampled on April 6, 1982. The victims of her village complained of vomiting, fever, headaches, backaches, swollen eyes, and chest pains. Both T-2 and HT-2 were found in her blood (FS736-21 and 44).

Another Hmong refugee in Thailand was treated and his blood was sampled on March 31, 1982. He had been exposed to a chemical attack on March 17, 1982, in Phou Bia, Laos. The agent which "looked like yellow rain" was sprayed from a helicopter at low altitude. The victim developed stomach and chest pains and vomition. Vomiting with blood developed and continued for 24 h. Other symptoms included headache, shortness of breath, dizziness, eye irritation, vision disturbances, rash, and blisters. Both T-2 and HT-2 were found in the blood of FS736-24 and T-2 in FS736-23.

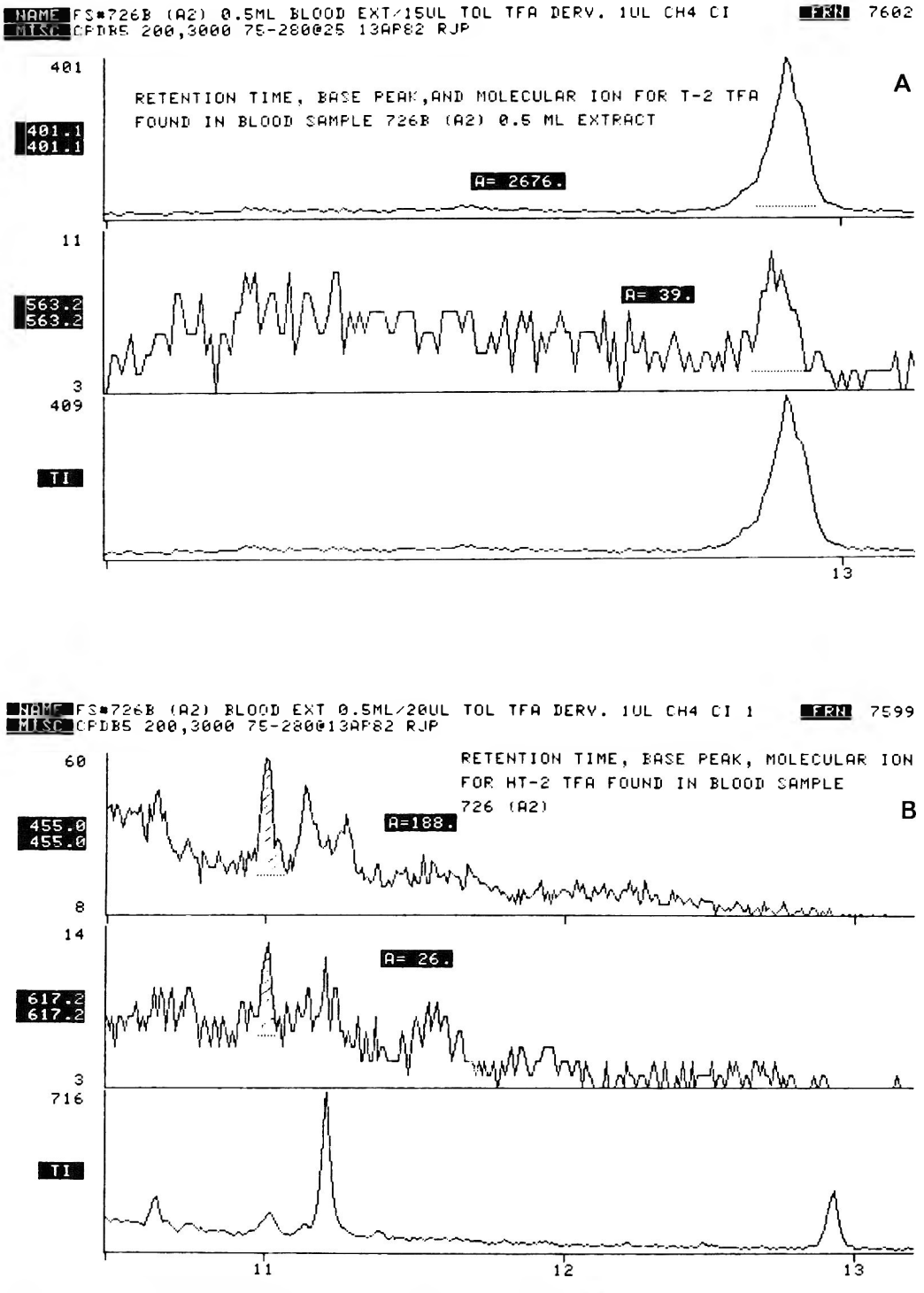

Figure 7. (A) Analysis of T-2 toxin in whole blood sample (FS72B = A-2) by positive chemical ionization in methane. Base peak at m/z$^+$ 401 of trifluoracetate ester of T-2 toxin is found at retention time 12.9. (B) Detection of HT-2 toxin by selected ion monitoring in chemical ionization mode resolved on 30 m CPDB-5 covalently bonded capillary (0.25 mm) column. Base peak (455) and molecular ion (617) are shaded, and area under peak is integrated for quantitation. Blood samples are those of victim FS726B.

Table 6. Analysis of various body tissues from victims of chemical attack [a]

Sample No. FS731	Tissue	Amount, g	Toxins		
			DAS	T-2	HT-2
A	heart	7.9		–	1.2 ppm
B	esophagus	13.5		25.1 ppb	4.02 ppm
C	liver	9.5		–	–
D	kidney	10.4	2.55[b] ppm	6.8 ppb	–
E	lung	4.5		8.5 ppb	–
F	large intestine	5.3		88 ppb	9.6 ppb

[a] (–) = not detected. All analyses done by positive chemical ionization in methane.

[b] DAS used as internal standard was overwhelmed by endogenous DAS. Victim was treated in Nong Pru Hospital.

A small aircraft sprayed a white powder near Pailin, Kampuchea, on March 5, 1982. The next day 10 of a group of 15 people walked through the area and developed symptoms identical to those already described. A second artillery shell attack occurred on March 7, 1982; blood and urine samples were taken from 3 survivors on March 13, 1982. T-2 and HT-2 were found in the urine (FS736-H, FS736-I, and FS736-J). The blood of FS736-H and FS736-I also contained T-2 and HT-2.

Other samples of blood and urine from victims were positive for T-2 and HT-2, and, although the victims were not identified, the results are tabulated in Table 5. Twenty-five of the 43 blood samples were negative when analyzed for trichothecenes.

One of the victims of the February 13, 1982, chemical attack in Tuol Chrey, Kampuchea, died on March 16, about one month after initial exposure. The victim had made a brief recovery on March 12 and 13 but then suffered a relapse at which time he showed signs of a fever and jaundice, became anuric, lapsed into a coma, and died. Shortly before death, the victim vomited blood. An autopsy was performed and tissues (heart, esophagus, stomach, liver, kidney, lung, large intestine) were stored in formaldehyde and later analyzed. Gross and microscopic pathological examination of these tissues revealed damage consistent with that reported for trichothecene intoxication (20). The results of the analyses are shown in Table 6. The kidney contained copious amounts of diacetoxyscirpenol (2.55 ppm) whereas the heart and esophagus contained 1.2 and 4.02 ppm HT-2, respectively. The significance of this concentration in cardiac tissue will be treated in the *Discussion* section. T-2 toxin was found in the esophagus, kidney, lung, and large intestine. DAS was used as the internal standard and it may have masked its endogenous presence in tissues other than kidney, where the endogenous level overwhelmed the internal standard.

The partial mass spectrum of DAS (positive chemical ionization in methane) found in the kidney is shown in Figure 8-A; T-2 in the large intestine (Figure 8-B); and HT-2 toxin in the heart tissue (Figure 8-C).

Discussion

The finding of T-2, HT-2, and DAS toxins in blood, urine, and body tissues of alleged victims of chemical warfare in Southeast Asia provides compelling proof of the use of trichothecenes as nonconventional warfare agents. The presence of both T-2 and HT-2 in the whole blood and HT-2 toxin in the urine collected from victims 24 h and more after exposure is highly significant and establishes that the blood and urine can be used to monitor exposure of humans to trichothecene toxins. The most persuasive proof is the detection of DAS, T-2, and HT-2 in body tissues of a victim who had succumbed to such a chemical attack. The tissue load was exceedingly high, 2.55 ppm DAS in the kidney, 1.2 ppm and 4.02 ppm HT-2, respectively, in the heart and esophagus. The presence of such a relatively large amount of HT-2 toxin in the heart may have special significance as to target organs. T-2 causes tachycardia in laboratory animals as well as focal necrosis in cardiac tissue of cattle when administered at higher concentrations. It is conceivable that T-2 toxin may have an etiologic role in the "sudden death toxic syndrome" of the Hmong people exposed to chemical attacks in Laos and Kampuchea.

Only limited research concerned with elimi-

nation rates of the trichothecenes has been conducted. Ueno et al. (21) reported that orally administered fusarenon-x was rapidly distributed to the tissues and reached peak levels by 3 h after dosing. The kidney was believed to be the major organ of excretion. Matsumoto et al. (22) conducted studies with T-2 toxin which led her to conclude that the liver and biliary system

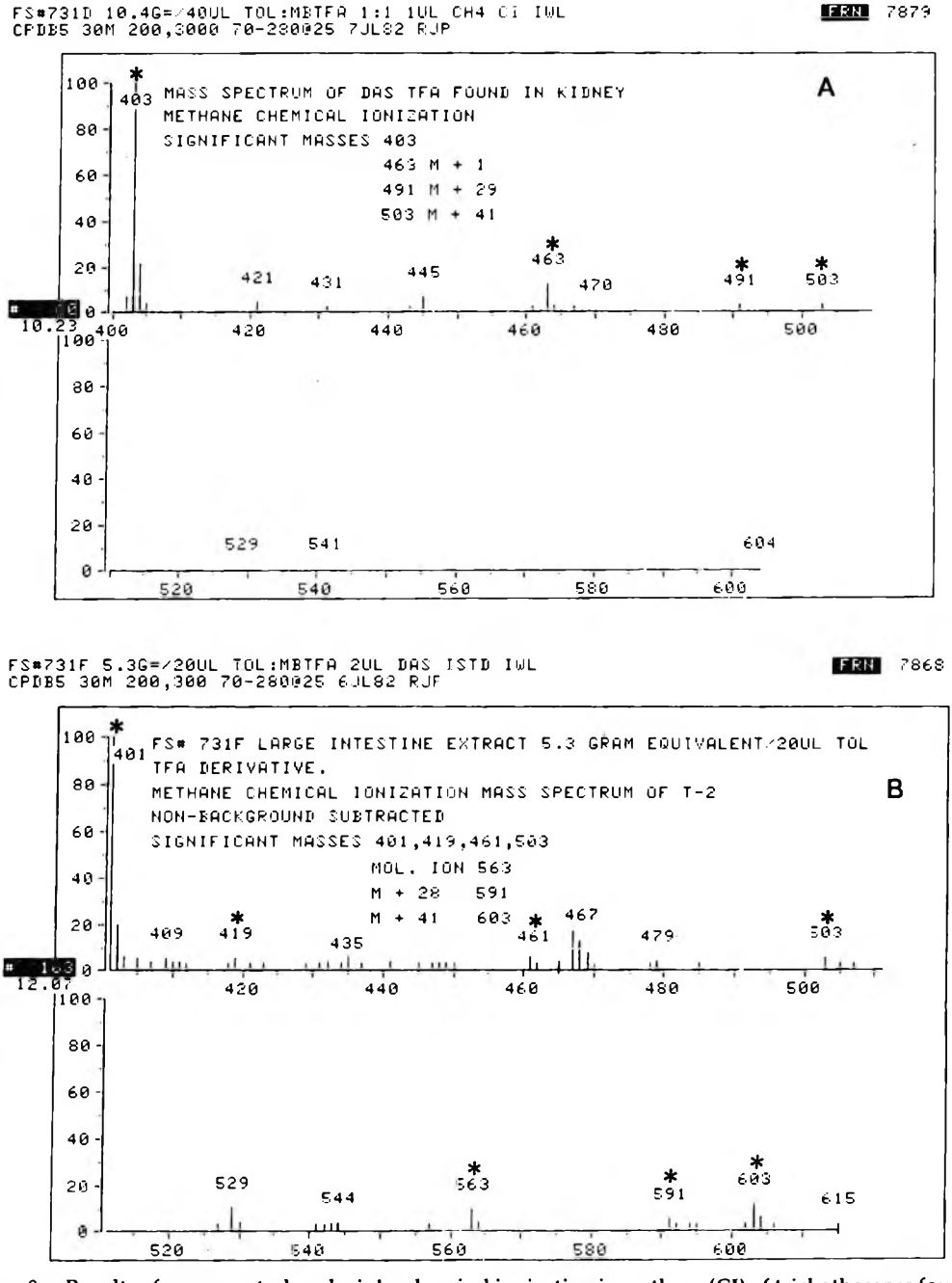

Figure 8. Results of mass spectral analysis by chemical ionization in methane (CI) of trichothecenes found in various tissues of victim of chemical attack of trichothecenes in Southeast Asia. Significant masses are marked with asterisks. (A) CI spectrum of DAS in kidney; (B) T-2 toxin in large intestine; molecular ion + 1 = 563, M + 29 = 591, M + 41 = 603; (C) HT-2 in heart.

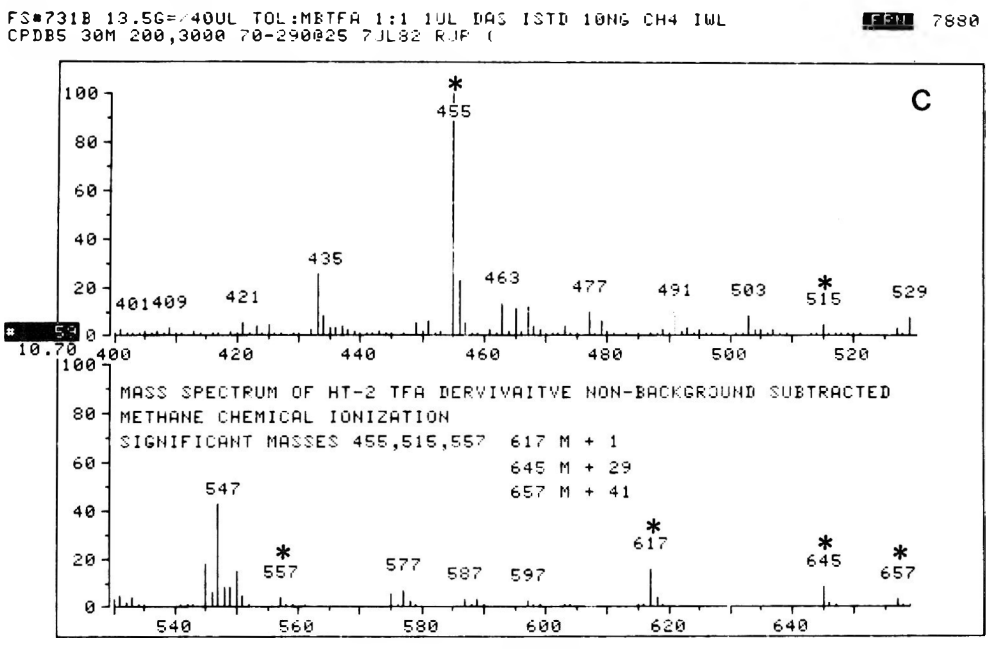

Figure 8. ***Continued***

were the major organs of T-2 excretion. Chi et al. (23) administered oral doses of T-2 to broiler chickens. Peak tissue levels were reached by 4 h after dosing, and the liver contained the greatest amount of toxin. By 12 h post-dosing, however, the muscle, skin, and bile contained the highest amounts of detectable toxin. By 48 h, 82% of the administered dose had been excreted. Robison et al. (24) showed that T-2 toxin was excreted into cow milk at levels up to 160 ppb after daily administration of 0.6 mg/kg doses.

Studies concerned with metabolism of the trichothecenes have also been limited in number. Yoshizawa et al. (25) reported that for in vitro studies with the S-9 fraction of rat liver homogenates, HT-2 made up 50% of the metabolic products. Other metabolites included TMR-1 (1996), TMR-2 (2%) and T-2 tetraol (4%). In in vivo studies, HT-2 was one of the major products eliminated in the excreta of chickens (26) as well as urine, feces, milk, and blood of dairy cattle (27).

The finding of T-2 toxin and HT-2 in the blood and tissue of humans weeks after their exposure to the toxins would seem to indicate that enough toxin remains bound in body tissues to allow detection by sensitive instrumentation. Trichothecenes have been shown to bind to ribosomal proteins (28) and to react with sulfhydryl-containing compounds such as glutathione (29) and with proteins such as albumin (30). It would appear that although most of the toxin would normally be expected to be excreted within 48 h after exposure, small amounts of the toxin and its metabolites remain bound to body tissues for much longer periods of time. The size of the dose administered and the route of exposure may have a significant effect on the proportion bound, since a sudden rapidly absorbed massive dose may overload normal excretion and detoxification mechanisms, resulting in greater tissue binding of the toxin. Moreover, the toxin may be absorbed by the kidney followed by slow release into the blood stream (31). Similar apparent long-term storage of mycotoxins has been reported previously for aflatoxin B_1. Although most of the administered dose of aflatoxin is rapidly metabolized, Shank et al. (32) demonstrated in studies in monkeys that unmetabolized aflatoxin B_1 could be detected up to 6 days after administration of a sublethal dose. Ochratoxin has also been found as residue in the circulating blood of swine (33).

Additional significant findings lie in the trichothecenes found in the leaf samples (T-2, DON, nivalenol) and yellow powder (T-2, DAS). The concentrations and their combination are not normally found in nature on the substrates described and it would appear that these mycotoxins found their way into the environment by

human intervention. The most compelling evidence is the presence of T-2 and DAS in the yellow powder. Both toxins are infrequently found in nature and rarely occur together. In our experience, copious producers of T-2 toxin (*F. tricinctum*) do not produce DAS, and conversely, good producers of DAS (*F. roseum* 'Gibbosum') do not produce T-2. This is also supported by our experience that a good producer of DON does not produce T-2 or DAS but could produce nivalenol. Thus, we have more than just the quantity of toxins produced to explain, but also the combinations produced by respective species and their isolates. Theoretically, it is possible to genetically manipulate or select an isolate that would produce copious amounts of 2 or more of these toxicants, but this would require a sophisticated research effort and sophisticated technology based on experience.

It is difficult to explain the presence of trichothecenes on leaf surfaces except by human intervention. *Fusarium* is not a leaf pathogen, so we would not expect it to colonize leaves indiscriminately. Certain species of *Fusarium* do colonize the roots and vascular tissue (causes wilt disease) of some plants and they would have to produce the toxins in situ and translocate them to the leaves. This has never been demonstrated in the pathogenesis of *Fusarium*-infected plants. If a pathogen like *F. oxysporum* f. *lycopersici*, pathogenic to tomatoes, were to produce trichothecenes and translocate them to the leaves, one would not expect such high concentrations and combination of toxins. Moreover, we are not certain that pathogenic isolates of the latter produce trichothecenes during pathogenesis. It is a well known plant pathological principle that production of toxins by pathogens in laboratory culture does not signify that these toxins also are produced in the host.

Apart from the controversy of the trichothecenes occurring on the leaves, it is difficult to imagine a reasonable explanation for the appearance of T-2 and DAS in the yellow powder. As to the claim that they dropped onto the soil and rocks from overhanging leaves, this is contrary to any known facts about trichothecene occurrence or distribution. The burden of proof remains with those presenting such an unlikely hypothesis.

The finding of T-2 toxin, diacetoxyscirpenol, deoxynivalenol, zearalenone, and *Fusarium* pigment in leaves, water, yellow powder, and fragments originating at sites of yellow rain attacks in Southeast Asia and their absence in background samples (leaves, corn, rice, water, soil) from areas not exposed to yellow rain strongly implicates their use as warfare agents. Moreover, the finding of T-2 toxin and HT-2 toxin (a metabolite of T-2 toxin in animals) in the blood, urine, and tissue of some victims of these attacks provides unequivocal proof of their use as weapons.

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FOOD ADDITIVES

Survey of Baby Bottle Rubber Nipples for Volatile *N*-Nitrosamines

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A local market survey of 27 baby bottle rubber nipples for volatile *N*-nitrosamines was conducted. *N*-Nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodibutylamine, and *N*-nitrosopiperidine were found at levels up to 387 ppb. All 4 nitrosamines migrated from the rubber nipples into water, milk, and infant formula when sterilized together in a manner typical of that used in the home. The source of the nitrosamines is the chemical accelerators and stabilizers added to raw rubber during the vulcanization process.

Several reports have been published recently describing the occurrence of volatile *N*-nitrosamines, a class of chemical carcinogens, in the atmospheres of rubber manufacturing plants (1–4). These compounds have also been found in new car interiors, especially in the areas around the spare tire (5, 6). These findings prompted investigations into the occurrence of nitrosamines in rubber products. Ireland et al. (7) identified 6 volatile nitrosamines in a variety of rubber products, including rubber baby bottle nipples, gloves, and condoms, at levels up to 1.4 ppm. Preussmann et al. (8) found nitrosamines in all 70 rubber nipples and pacifiers that they analyzed, at levels up to 230 ppb. In a preliminary study in our laboratory (9), 3 nitrosamines, nitrosodimethylamine (NDMA), nitrosodiethylamine (NDEA), and nitrosopiperidine (NPIP), were found in 2 types of nipples from one manufacturer at levels up to 281 ppb, and were confirmed by mass spectrometry (MS). In the same study, all 3 nitrosamines were found to migrate from a rubber nipple into milk or infant formula when sterilized together in a manner typically used in the home.

To obtain a more complete picture of the levels of nitrosamines in rubber nipples, we initiated a local market survey. This report describes the levels of nitrosamines found, and the extent of migration into milk, water, and infant formula during sterilization.

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Experimental

Reagents and Apparatus

Reagents and apparatus have been previously described (9). Water and methylene chloride were checked before use to ensure the absence of interfering peaks. A mixed nitrosamine standard containing 14 volatile nitrosamines (10) each at 0.5 μg/mL was prepared by serially diluting previously prepared stock solutions (2 mg/mL) with methylene chloride.

Determination

The method for the extraction of volatile nitrosamines from rubber nipples has been previously described (9). Briefly, the cut up nipple is soaked in methylene chloride (16–21 h). The nipple pieces are further extracted for 1 h in a Soxhlet extractor. Aqueous alkali is added to the methylene chloride extract, and the methylene chloride is distilled and discarded. A portion of the aqueous fraction is distilled and partitioned with methylene chloride, and the organic layer was dried, concentrated, and analyzed by gas chromatography (GC), using a thermal energy analyzer (TEA) as the detector. The lowest limit of measurement of the method is ca 1 ppb in a 5 g sample (one nipple).

Sterilized milk and infant formula were vacuum-distilled as previously described (10), with the exception that the distillates were extracted with three 15 mL portions of methylene chloride. Sterilized water was partitioned with three 15 mL portions of methylene chloride, and the organic layer was dried, concentrated, and analyzed by GC/TEA.

Results and Discussion

Analysis of a nipple by the methylene chloride extraction method described resulted in the extraction of greater than 80% of the nitrosamines present. Recovery of an internal standard, nitrosodipropylamine, added at the 10 ppb level to the methylene chloride extract of a rubber nipple before distillation, averaged 96%.

In a previous study (9), nitrosamine levels differed greatly from nipple to nipple, even

within the same lot, while halves of a single nipple contained the same levels. To facilitate comparison of nitrosamine levels in these products before and after sterilization, 2 nipples from the same package were each cut in half, one half of each nipple was exchanged, and the 2 new sets of nipple halves, comprised of half of each whole nipple, were used for experimentation and analysis.

Each nipple was sterilized according to its intended use. Those nipples designated for use with milk or formula were sterilized with the appropriate liquid by inverting the nipple halves on a glass bottle containing 50 g liquid, loosely capping, and placing in a boiling water sterilizer for 25 min. The nipple halves and liquid were subsequently analyzed for nitrosamines. Those nipples designated for use with water were boiled directly in water for 5 min in a 250 mL round-bottom flask fitted with a water-cooled condenser. The nipple halves were removed, and the nipple halves and water were analyzed. Those nipples not designated for a particular food use were sterilized both directly in water and with milk and infant formula.

Twenty-seven nipple samples were purchased at local retail markets in the Washington, DC area, or obtained from a local hospital. The samples represented 6 brands, both domestic and imported. The results of the nitrosamine analyses are shown in Table 1. Four nitrosamines were found: NDMA, NDEA, nitrosodibutylamine (NDBA), and NPIP. NDBA was found only in imported nipples, with levels ranging from 2 to 48 ppb. NDEA was the nitrosamine found most frequently (78% of the nipples analyzed) at levels ranging from 3 to 61 ppb. NPIP was found at the highest levels, ranging from 63 to 387 ppb, and an average of 148 ppb. NDMA ranged from 1 to 18 ppb. Four disposable (presterilized) nipples obtained from a local hospital were analyzed without further sterilization and contained NDMA at levels from 34 to 85 ppb (av. 63 ppb) (data not shown).

Several rubber nipple extracts were pooled and cleaned up on silica gel and acid Celite chromatographic columns as described previously (9) in an attempt to confirm the presence of the nitrosamines by GC/MS. NPIP was confirmed, but due to interferences and low nitrosamine concentrations in these samples, NDMA, NDEA, and NDBA were not confirmed. However, in our previous work (9), NDMA and NDEA were successfully confirmed by GC/MS in rubber nipple extracts. Also, an aliquot of a nipple extract containing NDBA was subjected to ultraviolet photolysis according to the procedure of Doerr and Fiddler (11). Re-analysis of the photolyzed extract by GC/TEA showed that 96% of the NDBA had been destroyed. This information, together with the specificity of GC/TEA towards nitrosamines, strongly suggests that NDBA was present in the rubber nipple.

Preliminary studies on rubber nipples from a single manufacturer had shown that nitrosamine levels were higher in nipples following sterilization (9), suggesting the presence of nitrosamine precursors. In this survey, no general increase was observed, although some samples displayed up to a 367% increase after sterilization. Other researchers have reported finding significant amounts of nitrosatable amines in rubber nipples (8), which, when heated in the presence of a nitrosating agent, could react to form nitrosamines.

In this study, all 4 nitrosamines identified in the rubber nipples migrated into water, milk, and infant formula upon sterilization. When rubber nipples were sterilized in water, levels of nitrosamines from 0 to 453 ng (av. 97 ng) migrated into the water, equivalent to an average migration of 41%. In milk and infant formula, nitrosamines were found at levels ranging from 0 to 46 ng (av. 14 ng) following sterilization with a rubber nipple. Nitrosamine migration into milk and infant formula was a function of the particular nitrosamine. The migration of NDMA, NDEA, and NDBA ranged from 0 to 38% (av. 17%), while for NPIP it ranged from 2 to 6% (av. 4%). Similar observations were made in our previous study (9). The low migration of NPIP may be due to solubility or steric interaction within the rubber matrix. No differences were observed between the average migration of nitrosamines into milk and infant formula. The average migration was, however, nearly 3 times greater into water than into milk and infant formula. This may be due to a more efficient extraction of the nitrosamines, facilitated by direct contact of the rubber nipple with the water during direct sterilization, or the heat resulting from direct water contact could have converted nitrosatable amines present in the nipple into nitrosamines, with subsequent extraction into the sterilizing water.

The source of nitrosamines in rubber nipples is the accelerators and stabilizers added to raw rubber during the vulcanization process (7, 8). Chemicals such as those shown in Figure 1 are added to introduce sulfur into the polymer, cross-linking the hydrocarbon chains, and thereby producing a polymer with the desired

Table 1. Volatile *N*-nitrosamines in rubber nipples, infant formula, milk, and water before and after sterilization

Nitrosamine, ppb, in rubber nipples								Nitrosamine, ng, in formula, milk, or water				Migration, %			
Unsterilized				Sterilized											
NDMA	NDEA	NDBA	NPIP	NDMA	NDEA	NDBA	NPIP	NDMA	NDEA	NDBA	NPIP	NDMA	NDEA	NDBA	NPIP
Infant Formula															
	8		63		9		65		10		11		24		3
	29		165		34		191		26		46		18		5
	12	47			11	48			10	9			17	4	
	27		114		27		133		26		37		19		6
	20		107		20		162		16		29		16		5
5	7			7	9			10	10			38	27		
2	16			1	13			5	10			9	12		
Milk															
	12		90		10		84		7		12		11		2
	4	8			3	7			6	ND[a]			29	0	
	11		79		12		87		11		18		18		4
2	11	2		1	4	2		2	3	3		18	5	29	
Water															
	8	29			5	26			14	50			38	38	
	13		81		12		103		22		75		34		18
18				16				54				47			
2	16	3		2	14	4		ND	51	ND		0	60	0	
	61		387		54		303		110		453		37		24
	17		155		18		125		68		279		41		19
	33		259		36		227		84		347		49		26
	7		74		8		98		30		147		44		20
14				15				55				45			
	24		189		24		209		44		230		37		25
4	3			9	11			13	29			40	134		
7	9			12	11			11	65			27	136		

[a] Not detected.

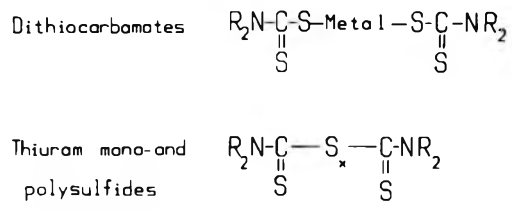

Figure 1. Examples of rubber accelerators.

elasticity, strength, and durability. Spiegelhalder and Preussmann (12) found up to 3.5 ppm nitrosamines as impurities in such accelerators. In addition, accelerators are designed to break apart during the vulcanization process, liberating sulfur. At the same time, secondary amines are also released, which can subsequently nitrosate. Nitrogen oxides could be acting as the nitrosating agent, particularly in the atmospheres of rubber manufacturing plants.

The fact that nitrosamines can be extracted from a rubber nipple into water suggests that a feeding infant will consume preformed nitrosamines. Also, work in our laboratory has corroborated that of Spiegelhalder and Preussmann (12), which showed that high levels of nitrosatable amines could be extracted from a rubber nipple into simulated saliva containing nitrite. Nitrosatable compounds consumed by the infant may react with nitrite present in the saliva, resulting in additional nitrosamine exposure.

The rubber industry is presently working on means for reducing or eliminating nitrosamines in rubber nipples. Spiegelhalder and Preussmann (12) have proposed several alternatives. Accelerators containing no amine functional groups could be used. Such chemicals would therefore not nitrosate. Alternatively, accelerators with amine functional groups, which, when nitrosated form weak or noncarcinogenic nitrosamines, could be used.

The German government established a regulation effective January 1, 1982, which permits a maximum of 10 ppb nitrosamines and 200 ppb nitrosatable compounds in rubber nipples and pacifiers, when determined by an artificial saliva extraction technique (12). We are presently monitoring progress in the reduction or elimination of nitrosamines in rubber nipples and will report our findings in subsequent papers.

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EXTRANEOUS MATERIALS

Brine Saturation Technique for Extracting Light Filth from Canned Crabmeat and Shrimp: Intralaboratory Study

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An intralaboratory study was made of a new technique for isolating light filth from canned shrimp and crabmeat. Recoveries of 93.8% for insect fragments and 89.4% for rodent hair plus the brevity and simplicity of the new technique make it superior to the 2 existing official methods for these products.

There are at present separate but somewhat similar official methods for filth in canned shrimp and canned crabmeat (1). Both methods give satisfactory recoveries but are more lengthy and involved than the brine method presented here. The principal shortcoming of the official methods is the use of protein-coagulating reagents, acid in the shimp method and isopropanol in the crabmeat method, both of which coagulate suspended protein, making filtration difficult. In addition, the extraction vessel used is a percolator, which requires considerably more wash water, thus adding to the volume that must be filtered.

The brine saturation method described here is a single, simple, streamlined alternative designed to eliminate the problems associated with the 2 official procedures. This study consisted of 12 samples each of canned shrimp and canned crabmeat. Each sample was spiked with 12 rodent hair fragments and 12 insect elytral squares and was examined only by the brine saturation procedure. Two analysts participated in the study.

METHOD

Transfer entire can contents (6–8 oz) to 1 L beaker; wash can and parchment, if present, with brine (made by dissolving 360 g rock salt or equivalent per liter of tap water and filtering). Mull with spoon to break up lumps and/or whole shrimp and fill to ≤900 mL with brine. Add magnetic stirring bar and heat to boiling with constant stirring on magnetic stirrer hot plate (2). Boil 3 min, then place beaker in cold water bath and let cool below 40°C. Transfer contents of beaker to 2 L trap flask (3) with brine and fill flask 3/4 full with ca 32°C tap water. Add 30 mL olive oil, place trap rod and plunger in flask, and complete filling flask with tap water to 1.5 cm from top. Stir entire flask contents with plunger for 30 s with circular up and down motion to thoroughly mix the oil into the contents but not so vigorously as to incorporate air into the mixture. As oil rises to the surface, stir gently and intermittently to prevent entrapment of oil by the product. Repeat gentle stirring for 5 min, then let contents remain undisturbed for 30 min. Trap into 250 mL beaker, rinsing neck of flask with hot tap water. Perform second trapping with 30 mL portion of olive oil and repeat trapping procedure. Trap into original 250 mL beaker, rinsing flask neck again with hot tap water. Place trappings on hot plate and heat until hot, then filter on ruled paper, rinsing beaker with hot tap water. After all liquid from trappings has passed through the filter, rinse beaker and filter funnel with isopropanol. Remove and examine filter papers at 30×.

Results and Discussion

Table 1 gives recoveries by product, collaborator, and filth element for each of the 48 determinations. The overall recovery of rodent hair was 89.4% and of insect fragments was 93.7%. These results represent an improvement over recoveries reported for the official procedures (4, 5). The brine technique is simple and rapid and allows easy completion of 12 samples in a single working day. The official method for crabmeat is reported (4) to take about 2 h/sample, excluding the reading of the papers. The shrimp method is reported (5) to take about 30 min/sample, not including filtration and examination. However, with the large volume of coagulated protein present in the trappings, this represents only a fraction of the time required for completion of the sample.

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Table 1. Recovery of rodent hair and insect fragments from canned crabmeat and shrimp (12 each added)

	Crabmeat				Shrimp			
	Analyst A		Analyst B		Analyst A		Analyst B	
Sample No.	Hair	Frag.	Hair	Frag.	Hair	Frag.	Hair	Frag.
1	11	12	9	11	10	11	12	12
2	11	12	10	12	8	9	11	12
3	11	11	12	12	7	11	12	11
4	11	12	11	11	10	11	11	11
5	12	10	11	11	12	12	11	12
6	12	11	10	12	12	10	12	12
7	12	12	10	10	11	11	12	12
8	12	9	14	13	10	12	12	12
9	11	11	11	12	12	12	12	11
10	12	11	7	9	8	12	11	11
11	12	12	12	12	4	11	9	11
12	11	9	11	12	9	11	11	11
Total	138	132	128	137	113	133	136	138
Av., %	96	92	89	95	78	92	94	96

Overall total hair = 515/576 = 89.4%
Overall total frag. = 540/576 = 93.8%

Recommendations

The data presented in this study clearly show that this technique is excellent in recovery and efficiency. It is recommended that the procedure be studied further as an AOAC interlaboratory study with the possibility of adoption as an official procedure, replacing the 2 existing methods. It is further recommended that studies on brine saturation filth extraction methods be continued and expanded to include other food and drug products.

Acknowledgment

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DRUGS IN FEEDS

Identification and Semiquantitation of Monensin Sodium in Animal Feeds by Thin Layer Bioautography

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A bioautographic technique for the determination of monensin sodium contamination in animal feeds is described. The feeds are extracted in aqueous methanol and the initial monensin extracts are isolated by filtration through an alumina column. These eluates are partitioned between 5% NaCl and methylene chloride, and are further purified through a Sephadex LH-20 column. A 10 mL eluate containing the monensin is collected from the Sephadex column and evaporated, and the residue is dissolved in methylene chloride. Aliquots are spotted on a thin layer plate and monensin is detected by a thin layer bioautographic technique, using *Bacillus subtilis* as the test organism. The reliable limit of sensitivity is 100 ppb, but 10 ppb can be detected. This technique can be used to semiquantitate monensin by comparing the zones of inhibition of unknown test samples against monensin standards.

Monensin, a relatively new antibiotic, was reported by Agtarap and Chamberlin (1) in 1967 to be a biologically active compound. Initially, monensin was found to be effective against coccidia in chickens (2) and, more recently, to increase feed efficiency and weight gain in beef cattle (3). However, at certain levels it is toxic to horses (4). Therefore a method is needed for the detection of low levels of monensin in animal feed.

Donoho and Kline (5) effectively used a thin layer bioautographic technique to determine monensin in animal tissues. We have investigated and modified the technique, which was first developed by Kline and Golab (6), and found that it could be adapted to assay animal feeds. The measurements of the zones of inhibition on bioautographs can be used to semiquantitate monensin sodium.

Published methods for detecting monensin are long and arduous, and use solvents such as carbon tetrachloride, pyridine, benzene, or others known to be carcinogenic. These methods can be used to detect monensin in feeds or tissue, but none can be used for both. The present method, as developed, can detect 10 ppb monensin in animal feeds, but results at this level are inconsistent. The solvents used are less hazardous to health and results can be obtained within 2 days.

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METHOD

Reagents

(a) *Solvents.*—Methanol and methylene chloride, distilled-in-glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

(b) *Alumina.*—80–200 mesh (Fisher Scientific Co., Fair Lawn, NJ 07410), for 75 mm column.

(c) *Sephadex LH-20.*—Particle size 25–100 μm (Pharmacia Fine Chemicals, Piscataway, NJ 08854), for 75 mm column.

(d) *Bacillus subtilis.*—ATCC 6633 or commercial spore suspension (1 mL vials, Difco, Detroit, MI 48232).

(e) *Monensin sodium reference standard.*—Label declaration, 960 μg/mg (Eli Lilly Laboratories, Greenfield, IN 46140). (*1*) *Stock solution.*—1000 μg/mL. Weigh 10.4 mg monensin sodium (or, if different label declaration, amount to give 1000 μg/mL), transfer to 10 mL volumetric flask and dilute to volume with anhydrous methanol. Store in dark, cool place. Discard after 30 days. (*2*) *Intermediate solution.*—10 μg/mL. Pipet 1.0 mL stock solution (*1*) into 100 mL volumetric flask and dilute to volume with methanol–water (8 + 2). Prepare fresh before each use. (*3*) *Working solutions.*—1.0, 0.5, and 0.1 μg/mL. Pipet 10, 5, and 1 mL of intermediate solution (*2*) into individual 100 mL volumetric flasks and dilute to volume with methanol–water (8 + 2). Prepare fresh before each use.

(f) *Bioautography media.*—To prepare bioautography medium No. 2 (5), dissolve 0.69 g dibasic potassium phosphate (J.T. Baker Chemical Co., Phillipsburg, NJ 08865), 0.45 g monobasic potassium phosphate (J.T. Baker), 10 g anhydrous glucose (dextrose) (Difco), and 2.5 g yeast extract (Difco) in water and dilute to 1 L with water. Just before bioautography prepare, by the

method of Kline et al. (7), medium No. 2 plus 1.5% purified agar (Difco), medium No. 2 plus 0.6% purified agar, and 1.5% Nobel agar (Difco).

(g) *2-(4-Iodophenyl)-3-(4-nitrophenyl-5-phenyl) tetrazolium chloride (INT dye).*—Weigh 200 mg INT dye (J.T. Baker) into 100 mL volumetric flask. Dissolve in 20 mL methanol and dilute to volume with water. Store in refrigerator.

(h) *Color reagent (8).*—Dissolve 3 g reagent grade vanillin in 50 mL methanol. Add 0.5 mL concentrated H_2SO_4. Mix by swirling and dilute to 100 mL with methanol. Mix well and store in amber glassware in dark. Prepare fresh daily.

Apparatus

(a) *Chromatographic tubes.*—200 × 300 mm, fitted with coarse porosity fritted glass disk and Teflon stopcock for Sephadex LH-20 column; 20 × 200 mm Chromaflex tube (Kontes Co., Vineland, NJ 08360) for alumina column.

(b) *Rotary evaporator.*—Rotavapor R110 vacuum evaporator (Brinkmann Instruments, Inc., Westbury, NY 11590), water bath temperature 48–50°C.

(c) *Receiving flasks.*—250 mL pear-shaped flasks and 10 mL concentrator tubes, with ₹ 19/22 ground glass stoppers (K-570050, Kontes Co.).

(d) *Analytical evaporator.*—N-EVAP (Organomation Associates, Inc., Northborough, MA 01532), water bath temperature 48–50°C with nitrogen flowing into each tube.

(e) *NUNC Bioassay dish.*—243 × 243 × 18 mm disposable dish (Vanguard International, Inc., Neptune, NJ 07753).

(f) *Thin layer chromatographic (TLC) plates.*—20 × 20 cm precoated with silica gel G, 250 μm thick (Uniplate, Analtech Inc., Newark, DE 19711) or K-6 Whatman (Whatman Laboratory Products Inc., Clifton, NJ 07014). The Uniplate is recommended for bioautography.

(g) *Incubator.*—Circulating air, 37°C.

(h) *Glass TLC developing chamber.*

(i) *Paint-spray atomizer.*

Column Preparation

(a) *Alumina adsorption.*—Place glass wool plug at base of Chromaflex tube. Add alumina to height of 75 mm. Tap gently and add more alumina if necessary. Place small amount of glass wool over alumina to ensure uniform flow. Keep column dry until used. Do not restrict flow.

(b) *Sephadex LH-20.*—Weigh 6.82 g Sephadex LH-20 into 150 mL beaker. Add 75–100 mL methanol–water (8 + 2). Let equilibrate 4 h and then pour total contents into 20 × 300 mm chromatographic column. Do not restrict flow. Wash beaker with additional methanol–water (8 + 2) to ensure complete transfer of Sephadex. Wash column with methanol–water (8 + 2) to make surface of Sephadex uniform. Retain at least 2 cm solvent above column bed.

After each use, clean Sephadex column with 80–100 mL anhydrous methanol. Regenerate Sephadex with 80–100 mL methanol–water (8 + 2), allowing volume to elute to 2 cm above column bed. Let equilibrate 1 h.

Sample Preparation

Weigh 10 g finished feed into 150 mL plastic centrifuge bottle with screw cap. Add 50 mL methanol–water (8 + 2) and shake vigorously 30 min on mechanical shaker. Centrifuge mixture 15 min at 1500–1800 rpm. Repeat extraction procedure. If concentration of monensin is less than 1.0 ppm, decant both 50 mL extracted portions directly into alumina column and elute. Collect eluate in 250 mL separatory funnel and add 100 mL 5% NaCl. If concentration is greater than 1.0 ppm, decant into alumina column and elute. Transfer aliquot equivalent to 0.100 μg monensin to separatory funnel and add 100 mL 5% NaCl. Shake vigorously and let mixture stand 5 min. Partition monensin into three 30 mL portions of methylene chloride; after addition of each 30 mL, shake funnel and then let methylene chloride layer separate. Drain lower layer into 100 mL pear-shaped flask. Combine methylene chloride portions and evaporate to dryness in rotary evaporator with water bath at 48–50°C.

Dissolve residue in 1.0 mL methanol–water (8 + 2) and transfer to Sephadex LH-20 column. Let solution just enter bed of column. Begin collecting eluates immediately. Wash flask with two 3.5 mL portions of methanol–water (8 + 2). Let each portion enter bed of column. Add additional 10 mL solvent to flask as final wash. Collect 18 mL eluate and discard. Add final 10 mL portion of solvent and collect this fraction, which should contain monensin, in 10 mL concentrator tube. Evaporate to dryness in analytical evaporator, with water bath at 48–50°C and nitrogen flowing into tube. Process blank control and spiked sample with each set of experimental samples.

Thin Layer Chromatography

Place 100 mL methylene chloride-methanol (9 + 1) into developing chamber lined with filter

paper. Let chamber equilibrate 30 min.

Add 1.0 mL methylene chloride to each extracted sample in evaporator tube and swirl gently to dissolve residue. Spot equivalent of 0.1 μg monensin 3 cm from bottom of TLC plate. Place plate in developing chamber and let solvent front rise 18 cm from bottom. Remove plate and air dry 1.5–2 h for bioautography or 10–15 min for TLC.

Bioautography

Pour 100 mL 1.5% Nobel agar into bioassay dish to anchor TLC plate and let agar solidify 30 min. Fix TLC plate by spraying with 30 mL medium No. 2 with 1.5% purified agar, using an atomizer. (Reagent sprayers will not spray agar because of its thickness; therefore, a paint sprayer, powered by laboratory air supply, is recommended.) Let agar set 1.0 min. Place TLC plate centrally and as firmly as possible on Nobel agar. Cool 100 mL medium No. 2 with 0.6% purified agar to 50°C, and then inoculate with 1.0 mL *B. subtilis* suspension. Swirl gently to mix, avoiding formation of bubbles, and pour evenly over surface of TLC plate. If bubbles form on surface, burst them with hot needle. Cover plate and let contents set for 15–20 min. Place plate in incubator with circulating air and incubate 16–18 h at 37°C.

After incubation, spray INT dye uniformly over surface of TLC plate; use reagent sprayer powered by laboratory air supply and spray in well ventilated hood or special spray cabinet. A red color will develop after 15 min. Compare R_f values and zones of inhibition of test samples against monensin sodium reference standards.

Results and Discussion

Recovery determinations were conducted with the bioautography technique by using 10 g portions of blank feed spiked with 1.0, 0.5, and 0.1 μg monensin (Table 1). Recoveries were reproducible at the lowest level (0.1 μg) and were estimated to be 80–90%. TLC was the initial method used to identify monensin; the plates were spotted, developed, and sprayed with a methanolic vanillin color reagent according to the method of Golab et al. (8). The intensity of the red color of monensin was proportional to its concentration, and at levels greater than 1.0 ppm, the reaction was semiquantitative. Results were obtained by comparing the unknown test samples against reference standards. At levels less than 1.0 ppm the spots were diffused and lighter in color. TLC plates spotted with more than 1.0 ppm monensin had brighter red spots, which when viewed under ultraviolet (UV) light appeared as bright orange. After the TLC plate was sprayed a second time and viewed again under UV light after 1–5 min, the orange spots appeared to be brighter. These plates were used to determine sensitivity by comparing both methods of detection.

Table 1. Recovery data comparing 9 animal feed samples, 3 each spiked with 0.1, 0.5, and 1.0 ppm monensin sodium, for semiquantitative determination by bioautography

Sample No.[a]	Vol. spotted, μL	R_f × 100	Zone diam., mm[b]	Est. rec., %[c]
Blank	100	—	—	—
1	100	60	5	100
2	100	57	4	80
3	100	58	5	100
4	20	60	3	60
5	20	58	4	80
6	20	59	5	100
7	10	63	6	120
8	10	62	6	120
9	10	58	5	100
Ref. std, rec.[d]	10	59	5	100
Ref. std[e]	10	60	5	—

[a] Blank, feed sample without added monensin; Samples 1–3, 0.1 ppm; Samples 4–6, 0.5 ppm; Samples 7–9, 1.0 ppm.

[b] Zone diameters obtained by using a sample spotting and quantitating template.

[c] Recovery estimates based on TLC determination, assuming 100% resolution of reference standard.

[d] Reference standard recovery sample, 0.1 μg monensin sodium/mL carried through procedure starting with 5% NaCl-methylene chloride partition.

[e] Reference standard, 0.1 μg monensin sodium/mL directly from dilution flask.

At levels less than 1.0 μg monensin, the bioautography technique showed sensitivity; therefore, this technique was used to semiquantitate monensin by comparing the zones of inhibition of test samples to those of the reference standards. The sensitivity of the method was determined to be 0.1 μg. Recovery data are presented in Table 2.

When feeds spiked at levels of 1.0 μg monensin were analyzed, other spots did develop on the bioautography plate, but the R_f values were less than the value for monensin. The R_f values for monensin on TLC plates (K-6) ranged between 0.65 and 0.70, and on the bioautography plates (Uniplates) ranged between 0.58 and 0.63.

Other antibiotics used as preventative medication for broilers and beef cattle were tested in combination with monensin in the TLC procedure with K-4 Whatman plates. These included

Table 2. Recovery data of animal feed samples, each spiked with 0.1 ppm monensin sodium, for semiquantitative determination by bioautography

Sample No.[a]	R_f × 100	Zone diam., mm[b]	Est. rec., %[c]
Blank	—	—	—
1	60	5	100
2	57	4	80
3	58	5	100
4	60	5	100
5	59	5	100
6	59	5	100
7	59	5	100
8	59	6	120
9	58	5	100
10	57	4	80
Ref. std, rec.[d]	57	4	80
Ref. std.[e]	58	5	

[a] Blank, feed sample without added monensin.

[b] Zone diameters obtained by using sample spotting and quantitating template.

[c] Percent recovery estimates based on TLC determinations, assuming 100% resolution of reference standard.

[d] Reference standard recovery sample, 0.1 μg monensin sodium/mL carried through procedure starting with 5% NaCl-methylene chloride partition.

[e] Reference standard, 0.1 μg monensin sodium/mL spotted directly from dilution flask. Volumes spotted, 100 μL for feeds and 10 μL for standards.

lincomycin, zinc bacitracin, flavomycin (bambermycin), chlortetracycline (HCl), oxytetracycline, lasalocid sodium, and tylosin. The antibiotics were mixed together, extracted, and tested for intereference. None interfered with monensin; however, tylosin gave identifiable R_f values ranging between 0.45 and 0.50 and lasalocid sodium gave values between 0.73 and 0.75. Lasalocid sodium and tylosin gave distinct color differences from monensin when the plate was sprayed with the vanillin color reagent. Chlortetracycline and oxytetracycline remained at the origin.

Although the TLC technique can provide results within the same day of extraction, the sensitivity is not lower than that obtained with bioautography. Results cannot be obtained as quickly with bioautography, but the sensitivity tested to 10 ppb. This modified technique can be used as a screening test for monensin and also as a semiquantitative method.

An investigation has been initiated to adapt the procedure for the detection of monensin in animal tissues, and a high-pressure liquid chromatographic quantitation method for monensin is in the preliminary stages of development.

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MICROBIOLOGICAL METHODS

Freeze-Dried Mixed Cultures as Samples for Proficiency Tests and Collaborative Studies in Food Microbiology

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A method is presented for preparing samples of freeze-dried mixtures of microorganisms for proficiency tests and collaborative studies. The samples may include most microorganisms that are found in routine analysis in food laboratories. Transport of samples during 48 h did not decrease the number of microorganisms, nor was the variability among samples significantly affected by transport. The standard deviation of counts after 5 weeks of storage varied from 0.04 (*Staphylococcus aureus*) to 0.17 (*Clostridium perfringens*) log unit. Storage of samples for 10 weeks decreased the number of viable organisms by 0.02-0.43 log unit. Variability among samples increased for *Providencia alcalifaciens* and *Bacillus cereus* after 10 weeks of storage. No significant increase was found for the other organisms.

Proficiency testing of laboratories has been carried out during the last 40 years. Testing started as an informal interchange of samples between clinical laboratories, but has since developed into more formal programs (1). Proficiency testing is now a regular activity in clinical analytical work (2). However, this kind of quality assurance has not been common in microbiological food laboratories, possibly because of difficulties in preparing appropriate samples. Unlike clinical laboratories, microbiological food laboratories carry out quantitative analyses, which places certain requirements on the samples used. Thus, the numbers of microorganisms in the samples must not change within a reasonable period of time, and the variation among samples must be low. In addition, it is an advantage if many different kinds of microorganisms can be included in the same sample. These requirements are difficult to satisfy if food samples are used. Thus, when starting to prepare proficiency testing samples at the Swedish National Food Administration (SNFA), we decided to use freeze-dried mixtures of microorganisms.

Freeze-dried pure cultures of bacteria have been used by Gavan (3) in a quality evaluation program. However, the samples were used to check the ability of clinical laboratories to identify microorganisms. Cada (4) used freeze-dried samples of *Escherichia coli* in a proficiency test of water laboratories, comparing the most probable number technique, the membrane filter method, and standard plate count.

This report describes a method of preparing freeze-dried mixtures of microorganisms as samples for proficiency testing of microbiological food laboratories. These samples may also be used in collaborative studies in food microbiology.

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Experimental

Organisms

Most bacteria used in this study (Table 1) were previously isolated from foods at our laboratory. The remaining strains originated from the SNFA laboratory culture collection. *Candida albicans* was obtained from the Centralbureau vor Schimmelcultures, The Netherlands. Before use, the identity of all bacterial strains was established by methods described by Cowan and Steel (5). Stocks of the cultures were maintained on brain heart infusion (Difco) agar slants and stored at 6°C.

To determine bacterial numbers before mixing the cultures, all strains were grown in brain heart infusion broth for 18 h at 37°C and numbers were determined on tryptone soya agar (Oxoid).

Samples

Freeze-drying media.—(*1*) 0.01M phosphate buffer: Dissolve 1.78 g $Na_2HPO_4 \cdot 2H_2O$ and 1.36 g KH_2PO_4 in 1 L water. BSA: Dissolve 5 g bovine serum albumin (Sigma fraction V Powder, A 4503) in 100 mL 0.01M phosphate buffer, and adjust pH to 7.3 with 2M NaOH. Filter solution through 0.22 μm filter (Millipore, Millex-GS).

(2) SPG: Dissolve 164 g saccharose, 1.40 g sodium glutaminate, 1.04 g KH_2PO_4, and 2.50 g K_2HPO_4 in 1 L water. Autoclave solution 15 min at 121°C. Freeze-drying medium consists of SPG and BSA mixed 1:1.

Table 1. Organisms in 2 samples of freeze-dried mixed cultures for proficiency tests and collaborative studies

Mixture 1		Mixture 2	
Organism	Strain	Organism	Strain
Providencia alcalifaciens	SLV-45, API 31	*Pseudomonas maltophilia*	SLV-41
Bacillus cereus	SLV-56, ATCC 14579	*Escherichia coli*	SLV-60
Citrobacter freundii	SLV-7	*Clostridium perfringens*	SLV-2
Staphylococcus aureus	SLV-44	*Salmonella poona*	SLV-103
Streptococcus faecium	SLV-39		
Candida albicans	J 8		

Sample preparation.—A tube or a flask with brain heart infusion broth was inoculated with a colony of the appropriate strain and incubated for 18 h at 37°C. Cultures were washed twice in 0.1% peptone water. They were then either suspended directly in the freeze-drying medium or placed in 0.1% peptone water if dilution was necessary before mixing the cultures. In the latter case, serial dilution was performed in 0.1% peptone water and then in the final step directly into SPG. Appropriate quantities from each culture were then transferred to a small flask. BSA was added to adjust the concentration of SPG and BSA to 1:1 in the sample to be freeze-dried. The culture mixture was carefully blended and 0.40 mL aliquots were distributed into sterile glass ampules (110 × 8.25 mm), using a syringe with a 1.3 × 110 mm needle.

Freeze-drying.—Freeze-drying was performed on an Edwards freeze-drier, Model EF03. Samples were frozen by a quick reduction of the pressure to 60 Pa. Freeze-drying was continued for about 5 h until a pressure of 8 Pa was achieved. Ampules were flame-sealed and checked for vacuum with an Edwards high frequency tester, Model 500K, D055-16-000. Samples were stored at room temperature in the dark before reconstitution.

Reconstitution of samples.—Ampules were washed out with four 1 mL portions of 0.1% peptone water, and washings were transferred to a sterile tube. Sixteen mL of 0.1% peptone water was added and the mixture carefully blended. The total volume of the samples was 20 mL.

Analysis

Samples were serially diluted in 0.1% peptone water. Numbers of *Providencia alcalifaciens* and *Pseudomonas maltophilia* were determined by aerobic plate count (APC), using plate count agar (Oxoid) incubated 72 h at 30°C. Numbers of *Citrobacter freundii* and *E. coli* were determined by coliform plate count with violet red bile agar (Oxoid) incubated 24 h at 37°C. Numbers of *Clostridium perfringens* were determined using egg yolk-free SFP agar base (Difco) containing 400 mg cycloserine/L. Numbers of *Bacillus cereus, Staphylococcus aureus, Streptococcus faecium* (Enterococci), and *Candida albicans* (yeasts) were determined by spreading aliquots of the sample onto the surface of blood agar (Oxoid) containing 5% young beef blood, Baird-Parker medium (Oxoid), Slanetz and Bartley medium (Oxoid), and Sabouraud dextrose agar (Oxoid) containing chloramphenicol (100 mg/mL) and chlortetracycline (100 mg/L), respectively. The blood agar and the Baird-Parker medium were incubated 48 h at 37°C, the Slanetz and Bartley medium 24 h at 44°C, and the Sabouraud dextrose agar 120 h at 22°C. The presence of *Salmonella* was determined by adding 1 mL undiluted sample to 9 mL tetrathionate broth base (Difco) containing iodine and brilliant green (6). After incubation for 24 h at 43°C, a loopful of the enrichment broth was spread on xylose lysine dextrose agar (Oxoid) and on brilliant green agar (Oxoid) and then incubated 24 h at 37°C.

Transport Study

Sixteen ampules from 2 mixtures (Table 1) were sent by mail to Lund in southern Sweden (about 600 km). The recipient returned the samples immediately to SNFA. These mailed samples spent 2 days in the mail system. Laboratory samples were stored at room temperature in the dark at the Biology Laboratory at SNFA.

Numerical Analysis

Bacterial counts were transformed to $\log_{10}$ values. Statistical evaluation of the results was carried out by methods described by Stearman (7).

Results

Effects of Transport

Mailed samples and laboratory samples were analyzed in parallel 5 weeks after freeze-drying (Tables 2 and 3). Comparison of mean counts by the *t*-test did not show any statistically significant differences between the 2 sets of samples ($P > 0.05$), except for *P. alcalifaciens* (APC) in mixture 1 ($P < 0.05$). However, the difference between mean counts of *P. alcalifaciens* was negligible (0.05 log unit) and, because the mean count of the mailed samples was higher than that of the laboratory samples, transport did not affect the mailed samples negatively.

The presence of *Salmonella* was demonstrated in all samples of both categories. Because this analysis is only qualitative, a 3-tube most probable number determination was carried out on one mailed and one laboratory sample to obtain a rough quantitative estimate. Numbers of *Salmonella* were 23 and 9/g, respectively.

The variability of mailed and laboratory samples was compared by the *F*-test. Transport did not increase the variance of the mailed samples significantly compared with the laboratory samples ($P > 0.05$).

Effects of Storage

Ten weeks after freeze-drying, another 14 samples from mixture 1 and 12 samples from mixture 2 stored at the laboratory were analyzed (Tables 2 and 3). When compared with the laboratory samples analyzed 5 weeks after freeze-drying, storage resulted in slightly lower mean counts for all organisms (0.02–0.43 log unit). The difference between mean counts after 5 and 10 weeks was significant ($P < 0.05$) for *P. alcalifaciens*, *S. faecium*, *P. maltophilia*, *E. coli*, and *C. perfringens*.

The variability in counts of *P. alcalifaciens* and *B. cereus* had increased significantly after storage for 10 weeks compared with that after 5 weeks storage ($P < 0.05$).

Salmonella was present in all samples of mixture 2 stored for 10 weeks.

Discussion

Variability

Bohdan et al. (8) concluded from a collaborative study that the standard deviation of aerobic plate counts within a single laboratory should not exceed 0.15 log unit in replicate samples. Based on results from their investigation on microbiological counts in frozen cod and on data in the literature (9, 10), Bohdan et al. (8) established

Table 2. Number of organisms (log_{10})/g in reconstituted culture mixture 1 determined at 5 and 10 weeks after freeze-drying

Analysis	5 weeks after freeze-drying				10 weeks after freeze-drying	
	Lab. samples ($n = 16$)		Mailed samples ($n = 16$)		Lab. samples ($n = 14$)	
	Mean	SD	Mean	SD	Mean	SD
Aerobic plate count	7.42	0.053	7.47	0.062	7.31	0.095
Coliform count	5.71	0.095	5.75	0.081	5.65	0.076
Bacillus cereus	3.28	0.095	3.34	0.085	3.20	0.190
Staphylococcus aureus	3.87	0.037	3.90	0.043	3.85	0.049
Enterococci	3.75	0.048	3.75	0.031	3.71	0.034
Yeast	4.88	0.049	4.84	0.049	4.85	0.049

Table 3. Number of organisms (log_{10})/g in reconstituted culture mixture 2 determined at 5 and 10 weeks after freeze-drying

Analysis	5 weeks after freeze-drying				10 weeks after freeze-drying	
	Lab. samples (n = 16)		Mailed samples (n = 16)		Lab. samples (n = 12)	
	Mean	SD	Mean	SD	Mean	SD
Aerobic plate count	5.97	0.099	5.95	0.083	5.85	0.093
Coliform count	3.93	0.095	3.88	0.154	3.55	0.081
Clostridium perfringens	5.62	0.144	5.59	0.173	5.19	0.175
Salmonellae	16 positive		16 positive		12 positive	

that split sample studies should not yield aerobic plate counts with standard deviations greater than 0.20. Similar results were obtained by Cada (4), who reported standard deviations between 0.034 and 0.354 for standard plate counts on 7 simulated water samples for proficiency tests. The variability of counts among samples in this study on freeze-dried mixed cultures was well below 0.15 log unit in most analyses.

Properties of Samples

Samples for proficiency tests and collaborative studies should preferably be of the kind normally handled by the participating laboratories. They should also be homogeneous, and the organisms to be determined should not be affected by transport. Food laboratories do not normally handle the kind of freeze-dried samples described in this report. On the other hand, these samples fulfill the requirements for homogeneity and stability, properties which can hardly be obtained with food samples. Moreover, these samples have the advantage, compared with food samples, of lacking a background flora that may be difficult to control.

At the SNFA, we have used freeze-dried mixed cultures as samples in proficiency tests of food laboratories in Sweden. Participating laboratories receive samples about 5 weeks after preparation and are instructed to do the analyses simultaneously during a specified period (1 week). It has been possible to include most organisms that can be found in the routine microbiological analysis of food in a single sample.

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DAIRY PRODUCTS

Determination of Lactose in Milk: Comparison of Methods

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A new enzymatic method based on the spectrophotometric measurement of reduced NAD$^+$ (β-nicotinamide-adenine dinucleotide) at 340 nm has been evaluated by comparing it with the AOAC copper reduction method. Ten samples of milk containing various levels of lactose were analyzed in duplicate by the enzymatic method, and 5 samples were analyzed by the copper reduction method. Statistical analysis using a factorial design revealed no statistical significance between results obtained by the 2 methods; recoveries were comparable, 97% or greater for all except one sample. There was no relationship between amount of lactose added and percent recovered.

There are two AOAC final action methods for the determination of lactose in milk, the polarimetric method (**16.055–16.056**) and the gravimetric method (**16.057**) (1). The polarimetric method requires a polarimeter equipped with a 400 mm tube, and the gravimetric method is time consuming and tedious. In addition, there is an AOAC first action infrared method (**16.058**) (1).

Several other spectrophotometric methods have been developed for this determination (2). The objective of this research has been to evaluate a new enzymatic method based on the spectrophotometric measurement of reduced NAD$^+$ (β-nicotinamide-adenine dinucleotide) at 340 nm (3). This method is highly specific, rapid, and simple to perform and the reagents are relatively safe compared with those used in other methods.

METHOD

Principle

Lactose is hydrolyzed to glucose and β-galactose in the presence of β-galactosidase and water. β-Galactose is then oxidized by NAD to galactonic acid in the presence of β-galactose dehydrogenase. The reactions proceed as follows:

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{glucose} + \beta\text{-galactose}$$

$$\beta\text{-Galactose} + NAD^+ \xrightarrow{\text{GAL-DH}} \text{galactonic acid} + NADH + H^+$$

The amount of NADH (β-nicotinamide-adenine dinucleotide, reduced) formed in this second reaction is stoichiometric with the amount of lactose, and is measured in a spectrophotometer at 340 nm.

Preparation of Sample

Protein is removed from the milk sample by precipitation, using Carrez reagents, as follows: Weigh ca 2 g milk into 100 mL volumetric flask and add ca 60 mL redistilled water (reagent grade I quality). Add 5 mL diluted Carrez I solution (3.60 g $K_4(Fe(CN)_6)\cdot 3H_2O$/100 mL, 5 mL Carrez II solution (7.20 g $ZnSO_4\cdot 7H_2O$/100 mL), and 10 mL 0.1M NaOH. Shake mixture vigorously, and let solution come to room temperature. Dilute to volume with redistilled water, and mix. Filter and use clear filtrate for assay.

Determination

Use either glass cuvets or disposable plastic cuvets (1 cm light path); the assay is carried out

Table 1. Determination of lactose (%) content in milk by enzymatic and gravimetric methods [a]

	Enzymatic			
Sample	A	B	Av.	Gravimetric
1	4.37	4.34	4.33	4.37
2	4.93	4.92	4.93	4.94
3	4.39	4.34	4.37	4.56
4	4.67	4.73	4.70	4.75
5	4.56	4.58	4.57	4.56
6	4.38	4.36	4.37	—
7	4.19	4.14	4.17	—
8	4.62	4.62	4.62	—
9	4.46	4.50	4.48	—
10	4.83	4.84	4.84	—

[a] Duplicates are Nos. 1 and 7, 2 and 10, 3 and 6, 4 and 9, 5 and 8.

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Table 2. Analysis of variance of enzymatic vs gravimetric methods

Source	Degrees of freedom	Sum of squares	Mean sq. (SS/DF)	*F*-value calcd	*F*-value from table 1%	*F*-value from table 5%
Method	1	0.00784	0.00784	2.54	21.20	7.71
Lactose level	4	0.41896	0.10474	33.8964	16.00	6.39
Method × level	4	0.01236	0.00309			

Table 3. Recovery of lactose by the enzymatic and gravimetric methods

Sample	Theor. lactose, %	Rec., % Enz.	Rec., % Grav.
1	4.37	—	—
2	5.04	98.60	98.02
3	4.47	98.76	102.13
4	4.85	97.01	98.04
5	4.66	99.02	97.96
6	4.47	98.76	
7	4.37	—	
8	4.66	99.25	
9	4.85	92.47	
10	5.04	96.80	

in the cuvet. Capillary pipets are recommended for volumetric transfers. Solution numbers refer to those in kit (available from Boehringer-Mannheim, Indianapolis, IN 46250). Carry out determination as follows:

Label one cuvet "blank" and one cuvet "sample." Pipet 0.20 mL solution 1 (pH 6.6 citrate buffer, 35 mg NAD, magnesium sulfate, and stabilizers), and 0.05 mL solution 2 (enzyme suspension β-galactosidase, ca 100 U) into each cuvet. Pipet 0.10 mL sample solution (filtrate) into sample cuvet. Use plastic stirrer to mix contents of both cuvets, and let stand 10 min at 20–25°C. Pipet 1.00 mL solution 3 (pH 8.6 potassium diphosphate buffer, 0.51 mol/L) into each cuvet. Add 2.00 mL water to blank cuvet, and 1.90 mL water to sample cuvet. Mix solutions in cuvets and let stand ca 2 min at 20–25°C.

Determine absorbances (A_1) of both solutions at 340 nm, using spectrophotometer with slit width of 10 nm or less. Add 0.05 mL solution 4 (enzyme suspension galactose dehydrogenase, ca 35 U) to each cuvet. Mix solutions, and let stand at 20–25°C until reaction has stopped (ca 10–15 min).

Determine absorbances (A_2) of both solutions. Note: If reaction has not stopped after 15 min, continue to read optical densities at 2 min intervals until absorbance increases constantly for 2 min.

Discussion

Five samples of milk containing various levels of lactose were prepared by adding lactose hydrate to warm milk (60°C) on a mechanical hot plate-stirrer; the equivalent levels of added anhydrous lactose ranged between 0.095 and 0.670%. Ten unknowns were prepared for enzymatic lactose analysis by splitting these 5 samples. In addition, aliquots of the 5 samples were sent to a commercial laboratory and analyzed for lactose by the AOAC gravimetric method.

Results of the analyses (Table 1) reveal a close correlation between the 2 methods; there was only one sample in which the difference exceeded 0.05%. Comparison of the duplicates analyzed by the enzymatic method also reveals close agreement. The difference exceeded 0.20% for only one pair of duplicates (Nos. 4 and 9). Statistical analysis of these data, based on calculated *F*-values, revealed no significant difference between the enzymatic and gravimetric methods (Table 2).

Lactose recovery rates were also determined for the 2 methods (Table 3). These data reveal that excellent recoveries were obtained by both methods; recovery was less than 96.80% for only one sample (No. 9). There was no direct relationship between the amount of lactose added and its recovery.

Finally, 4 replicates of one sample were analyzed to obtain more insight on the precision of the enzymatic method, with 2 replicates analyzed on 2 successive days. Results showed good precision. Overall, replicates yielded a result of 4.70% ± 0.04 (4.74, 4.70, 4.66, and 4.69%).

Results obtained by this study reveal that the enzymatic method yields results that agree closely with the AOAC gravimetric method. In addition, the close agreement of replicates indicates good precision. Thus, the enzymatic

method may be considered a highly specific, simple, rapid method for accurate and precise determination of lactose in milk.

An AOAC collaborative study of the method is in progress and will be the subject of a later report.

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FRUITS AND FRUIT PRODUCTS

Evaluation of Apple Juice Authenticity by Organic Acid Analysis

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An organic acid profile provides valuable information regarding authenticity of apple juice. The presence of D-malic acid is a clear indication of adulteration because this isomer does not occur naturally. Fumaric and citric acid levels above trace amounts are also inconsistent with pure apple juice; therefore, measurement of these organic acids may also be used as an authenticity check. Citric acid, total malic acid, and fumaric acid were determined in a single scan by liquid chromatography (LC) for 30 known pure apple juice samples. The L-isomer of malic acid was measured by an enzyme-specific method, and the D-isomer was calculated as the difference between total malic acid and L-malic acid.

Methods published by various authors (1–4) to verify the authenticity of apple juice relate to sugars, sugar ratio, stable isotope ratio, polyphenols, formol index, sorbitol, proline, ash, minerals, and organic acids. These tests together give useful information about the purity and quality of the juice. The organic acid scan developed in this laboratory, determines D-malic acid, L-malic acid, fumaric acid, and citric acid levels. Only the L-isomer of malic acid occurs naturally, so the presence of D-malic acid indicates adulteration with synthetic malic acid. Furthermore, an elevated citric acid level indicates adulteration, while a high level of fumaric acid indicates adulteration and/or overprocessing. Jeuring et al. (1) reported the determination of malic acid by liquid chromatography (LC) using a reverse phase column at room temperature. Junge and Spadinger (2) improved this method by using an ion exchange column, also at room temperature. In both methods, there was interference by an unknown compound eluting just before the malic acid peak. This made an accurate measurement of malic acid doubtful. Because D-malic acid is calculated as the difference between total malic acid and L-malic acid, great care must be exercised in each test to assure an accurate determination. Recent work in this laboratory has resulted in excellent resolution of malic, citric, and fumaric acids from interfering compounds.

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Experimental

Apparatus

Chromatography was performed with a Perkin-Elmer Series 2 liquid chromatograph, equipped with a Rheodyne Model 7125 syringe-loading sample injector and a Perkin-Elmer Model LC 75 variable wavelength spectrophotometric detector.

A Bio-Rad HPX-87 (300 × 7.8 mm) Aminex ion exclusion column for organic acids was used. The column was heated via a water jacket connected to a Tecam constant temperature water bath.

Operating conditions were mobile phase, 0.006N H_2SO_4; flow rate, 0.6 mL/min; wavelength, 207 nm; column temperature, 80°C.

Sample Preparation and LC Analysis

Samples of known pure apple juice were supplied by the Processed Apples Institute in Atlanta. Other pure samples were supplied by various donors in the apple juice industry. Samples were diluted to 3–5° Brix, filtered through a 0.45 μm Gelman filter, and injected in 5 μL aliquots. Concentrations of malic, citric, and fumaric acids were determined by comparing peak heights of samples and respective standards.

Enzymatic Determination

Enzymatic analyses for L-malic acid were carried out using the test method and reagents supplied in a commercial kit (Boehringer Mannheim, Indianapolis, IN 46250). Samples were diluted 1:100 and adjusted to pH 10. A Turner Model 350 spectrophotometer was used to monitor absorption at 340 nm. Disposable cuvets were used for all analyses.

Results and Discussion

The described method has been applied to both concentrated and single strength apple juice. Table 1 presents the organic acid profiles for 30 pure apple juice samples. Figure 1A is a typical chromatogram of a pure apple juice; Fig-

Table 1. Organic acid profile of known pure apple juices

No.	Identity	Total malic acid,[a] mg/100 mL	L-Malic acid,[b] mg/100 mL	Difference[c]	Fumaric acid, mg/L	Citric acid mg/100 mL
	Single strength juice:					
1	1980 Washington Red Delicious	280	290	−10	<1.0	ND[d]
2	1981 New York Idared	900	880	20	<1.0	ND
3	1980 New York Idared	770	780	−10	<1.0	ND
4	1981 Michigan Jonathan	520	500	20	<1.0	ND
5	1981 Washington Winesap	800	770	30	<1.0	ND
6	1980 Washington Red Delicious	430	430	0	<1.0	ND
7	1980 North Carolina Rome Beauty	330	320	10	<1.0	ND
8	1981 Massachusetts Cortland	530	560	−30	<1.0	ND
9	1981 Pennsylvania Stayman	550	560	−10	<1.0	ND
10	1980 New York McIntosh	500	500	0	<1.0	ND
11	Origin unknown	500	500	0	2.3	20
12	Origin unknown	510	520	−10	1.8	20
13	Origin unknown	360	360	0	<1.0	20
14	Origin unknown	370	400	−30	<1.0	10
15	Origin unknown	390	360	30	<1.0	<10
16	Origin unknown	410	380	30	3.2	10
17	Origin unknown	510	510	0	2.7	20
18	Origin unknown	370	370	0	<1.0	20
19	1981 Washington state	310	310	10	2.3	ND
	Juice from concentrate:					
20	Chilean	440	470	−30	0.9	ND
21	West German	480	500	−20	ND	ND
22	South African	520	520	0	ND	ND
23	Argentine	590	590	0	2.2	ND
24	German	600	610	−10	3.6	ND
25	Austrian	420	430	−10	3.0	30
26	Austrian	400	400	0	1.6	40
27	Argentine	540	540	0	3.7	10
28	Hungarian	800	800	0	4.3	30
	Single strength juice:					
29	Origin unknown	900	910	−10	2.4	<10
30	Origin unknown	220	220	0	3.0	10

[a] Determined by LC; coefficient of variation, 0.90%.
[b] Determined by enzymatic assay; coefficient of variation, 0.85%.
[c] Total malic acid minus L-malic acid.
[d] ND = not determined.

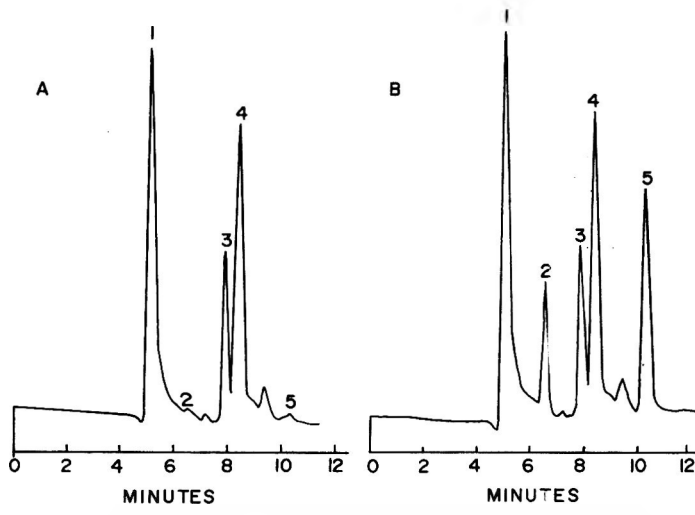

Figure 1. LC chromatograms of organic acids in apple juice. A, known pure sample; B, adulterated sample: 1, solvent front; 2, citric acid; 3, malic acid, 4, fructose and unknown; 5, fumaric acid.

ure 1B is a chromatogram of an apple juice with high citric and fumaric acid levels. The 3 compounds of interest, citric, malic, and fumaric acids, may be evaluated as shown below.

Citric Acid

Citric acid is present in pure apple juice at concentrations of not more than 10–20 mg/100 mL. Some European countries allow a maximum of 40 mg/100 mL (5). Concentrations higher than these values should be considered inconsistent with pure juice. Addition of citric acid or other fruit juices that have a higher citric acid content (pear, for example) is a possible source of adulteration (4).

Malic Acid

There is no D-malic acid in pure apple juice, so the analytical results for total malic acid by LC and for L-malic acid by the enzymatic method should be the same. Small errors can occur in routinely executed analyses for these compounds. Malic acid content of 30 known pure apple juice samples varied between 220 and 900 mg/100 mL (expressed on the basis of 14° Brix). An average difference of 0.3 mg/100 mL was observed between LC and enzymatic methods. According to *t*-test results, a difference of more than 16 mg/100 mL (1 standard deviation) indicates addition of D-malic acid ($P < 0.01$). Since the range of observed differences was ±30 mg/100 mL, we have established this as a practical value for the cutoff between experimental error and deliberate addition of racemic malic acid.

Fumaric Acid

Fumaric acid occurs as a by-product in the production of synthetic malic acid, and, in general, the presence of fumaric acid in apple juice in an indication of adulteration. Junge and Spadinger (2) reported that fumaric acid levels greater than 3 mg/L were not consistent with pure juice. However, this does not hold for juice from concentrate. Extremes in processing can result in the formation of fumaric acid without the addition of synthetic malic acid. For example, apple juice heated in closed vials for 3 days at 60°C did not produce fumaric acid. However, when the temperature was increased to 100°C and held an additional 3 h, as much as 10 mg fumaric acid/L was formed.

Further studies are needed to determine the cause of fumaric acid formation during the clarification and concentration processes, so that the level of fumaric acid may yield more definitive information as to whether adulteration and/or overprocessing has occurred.

Acknowledgment

The authors thank the Processed Apples Institute in Atlanta for obtaining the samples of authenic apple juice and supplying other valuable testing material.

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Determination and Mass Spectrometric Confirmation of Chloramphenicol Residues in Animal Tissues

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A rapid and sensitive liquid chromatographic (LC) method for determination of trace amounts of chloramphenicol (CP) in animal tissues has been developed. Chloramphenicol identity is confirmed by gas chromatography-mass spectrometry (GC-MS). CP can be routinely quantitated by LC at 10 ppb with 75% recovery, and GC-MS selected ion monitoring permits complete identification at concentrations as low as 5 ppb. Residues were determined in chicken tissues after intramuscular injection of CP. Depletion was rapid with no residues detected in muscle after 8 h, while 15-30 ppb CP was still determined and identified in the liver.

The broad spectrum antibiotic chloramphenicol (CP), D(−)-*threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1,3 propanediol, has been recommended for the treatment of *Salmonella* infections and for the prevention of secondary infections associated with chronic respiratory disease in poultry (1). CP is considered the most potent antibiotic for treating pneumonic and enteric conditions with respect to organism sensitivity, and is routinely and widely used in Europe (2). However, due to toxicological problems that have occurred in humans, e.g., toxicity of CP on bone marrow stem cells (3) and fatal but rare aplastic anemia, zero tolerance levels have been established. In countries like the United States, CP has never been authorized for use in food-producing animals, but illegal use of CP is a continuing problem, and tissue residues must be detected. Until now, the lack of sensitive and specific analytical methods has precluded the detection and identification of very low concentrations of CP in animal tissues in routine control as well as in metabolic studies. Bioassays are most often used (4) but their sensitivity is limited (0.5–1 ppm), and their precision appears to be variable; moreover, their specificity is questionable in samples (i.e., liver) which can contain interfering substances or even other antimicrobial agents. Nonspecific colorimetric assays have also been developed (5), often associated with a specific solvent extraction of CP. Their sensitivity is limited to 0.5–1 ppm, although some authors, using nonvalidated modified conditions, have claimed that a 0.1 ppm detection level could be reached (6).

Recently, we described an original liquid chromatographic (LC) method for determining CP residues in milk (7), and discussed the advantages of this technique over other chromatographic methods for CP analysis. This method could not be used for determinations in animal tissues. Modifications in the cleanup procedure and final analysis have resulted in an analytical method that is convenient for determining trace amounts of CP in muscle, fat, or liver. Furthermore, identification of CP by using gas chromatography-mass spectrometry (GC-MS) in the selected ion monitoring mode has been developed. The entire methodology has been applied to the determination and identification of CP residues in chicken muscle, fat, and liver at 4, 6, and 8 h following a single intramuscular administration.

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METHOD

Reagents

(a) *Solvents.*—Analytical grade ethyl acetate, isooctane (2,2,4-trimethylpentane), chloroform, propylene glycol, and methanol (E. Merck, Darmstadt, GFR).

(b) *Silylating reagent.*—*N,O*-Bis(trimethylsilyl)trifluoracetamide (BSTFA) (Pierce Chemical Co., Rockford, IL).

(c) *Analytical standard.*—Chloramphenicol (B grade) (Calbiochem, San Diego, CA).

(d) *CP stock standard solutions.*—(1) Dissolve 100 mg CP in 100 mL methanol (1000 ppm, solution A). (2) Dilute 10 mL solution A to 100 mL with methanol (100 ppm, solution B). (3) Dilute 10 mL solution B to 100 mL with methanol (10 ppm, solution C). (4) Dilute 10 mL solution C to 100 mL with methanol (1 ppm, solution D). (5) Just before use, dilute 10 mL solution D to 100 mL with distilled water (0.1 ppm, solution E).

Apparatus

(a) *Liquid chromatograph.*—Spectra-Physics (Santa Clara, CA) Model SP 8100, equipped with SP 8400 UV-visible detector set at 280 nm, and 225 × 2.1 mm id stainless steel column packed with 5 μm C_{18} Spherisorb ODS (Spectra-Physics). Operating conditions: isocratic mode with water-methanol (65 + 35) mobile phase pumped at ca 2500 psi to provide flow rate of 1 mL/min; UV photometer at 0.01 absorption unit full scale (AUFS); chromatogram recorded on 10 mV full scale recorder operated at 1 cm/min; injection of sample with 50 μL syringe in valve (Valco sample injector) equipped with 25 μL sample loop (Valco).

(b) *Gas chromatograph-mass spectrometer.*—Hewlett-Packard Model 5992B (Palo Alto, CA) quadrupole mass spectrometer with HP 5700A series gas chromatograph fitted with 12.5 m × 0.2 mm id OV-1 capillary fused silica column and HP 18947A capillary column interface. Operating conditions: temperatures—injection port 300°C, oven programmed from 190 to 220°C at 6°/min following initial 1 min hold at 190°C; helium carrier gas 0.8 mL/min. Hewlett-Packard magnetic tape software program (05992-10015, Peakfinder for Normalized Spectra) was used to calibrate and autotune mass spectrometer in electron impact mode at 70 eV. GC-MS system was operated in selected ion monitoring (SIM) mode. Using Hewlett-Packard 05992-10006 SIM tape program, 6 masses were selected in cycling time of 0.1 s.

LC Procedure

Grind 20 g tissue with 80 mL ethyl acetate for 3 min using Model PT-10-20 Polytron homogenizer. Add 10 g potassium carbonate, swirl to mix, then filter under vacuum through funnel fitted with fritted disk covered with Whatman No. 1 paper. Rinse homogenizer blades and jar using additional 40 mL ethyl acetate, and pour over filter. Place combined ethyl acetate filtrates in 250 mL separatory funnel and stir with 50 mL water. Let stand 30 min; then discard water phase. Transfer organic phase to a round-bottom flask and evaporate contents to dryness under vacuum. Add 80 mL acetonitrile saturated with isooctane and swirl flask to dissolve residue. Quantitatively transfer mixture to 125 mL separatory funnel and stir with 20 mL isooctane saturated with acetonitrile. Place lower phase in 100 mL round-bottom flask and evaporate under vacuum to small volume (a few mL). Quantitatively transfer to 5 mL conical flask, using acetonitrile, and evaporate to dryness. Dissolve residue in 0.25 mL chloroform, add 0.25 mL propylene glycol–water (50 + 50), mix with Vortex mixer, and let phases separate. Use aqueous alcoholic (upper) phase for LC analysis.

Determination.—Under conditions used, CP retention time is 9.4 min. As already shown (7), response is linear over 0–100 ng range. Determine CP concentration in sample extract by using standard curve and peak areas established from standard CP solutions as follows:

$$\text{CP, ppb (ng/g)} = C_0 \times S \times 0.25/S_0 \times W$$

where C_0 (ng/mL) = concentration of standard CP solution D; S_0 = peak area corresponding to standard CP solution D; S = peak area of sample; W = weight (g) of tissue sample.

Fortification.—20 g control muscle samples were spiked just before extraction, using 1, 3, and 4 mL solution E.

GC-MS Analysis

Following injection of propylene-water final tissue extract into liquid chromatograph, collect effluent manually at detector beginning at 9 min retention time, i.e., just before CP emerges from column to complete disappearance of CP peak. Evaporate collected mobile phase (about 1.5 mL) under vacuum, using absolute ethanol as water carrier. Dissolve residue in absolute ethanol and transfer to 1 mL PTFE-stopper conical flask. Evaporate solvent to dryness under nitrogen flow; add 20 μL BSTFA and let reaction proceed 1 h at 85°C. Then inject 5 μL into GS-MS system, using Ross injector. Monitor following 6 ions: 453, 451, 363, 361, 225, and 208 mass units.

Animal Study

Nine chickens were each given a single intramuscular injection of 20 mg CP/kg dissolved in propylene glycol–water mixture. Three animals were slaughtered 4, 6, and 8 h following administration of the drug to obtain sufficient tissues and organs to perform duplicate determinations. Liver, kidney and white muscle tissue and abdominal fat of every 3 birds were sampled and pooled, then ground. Two 20 g aliquots were weighed and then deep frozen at −30°C until analysis.

Results and Discussion

LC Determination

The chromatographic conditions specified here are the same as those proposed and discussed for the determination of CP residues in

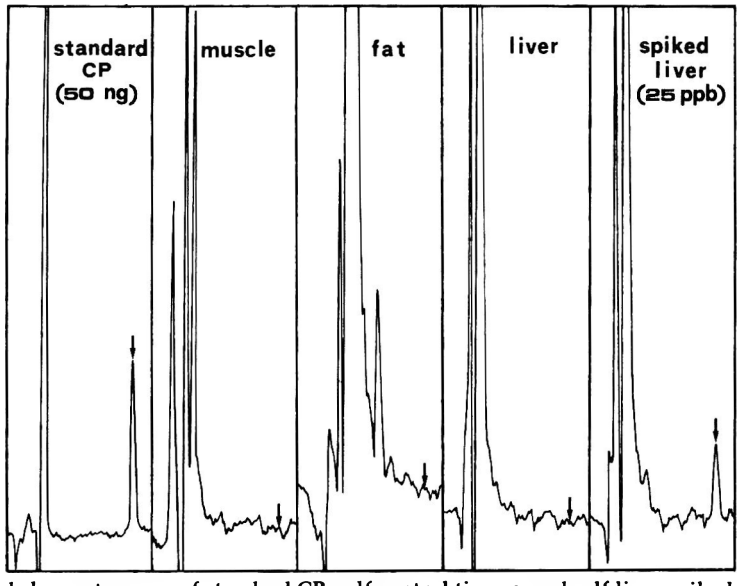

Figure 1. Liquid chromatograms of standard CP, calf control tissues, and calf liver spiked with 25 ppb CP.

milk (7). The eluting mixture is only slightly more polar (35% water instead of 30%) to allow complete elution of interfering substances before CP peak appearance. The extraction and clean-up procedures were adapted to eliminate tissue interferences during liver, muscle, and fat analysis. As shown in Figure 1, no interfering peaks occurred when calf control tissues were analyzed; average recoveries of CP from spiked calf muscle samples were in the range 70–80% (Table 1). No significant differences were observed for the different concentrations tested, even as low as 10 μg/kg. Similar satisfactory recovery and reproducibility was obtained in fat, kidney, and liver samples. The same results were obtained with chicken control tissues.

At the 10 ppb level, peak height is about 4 times the average baseline fluctuation. This value was chosen as the reliable detection limit for routine LC analysis. Setting the detector to 0.005 AUFS offered an additional means of discriminating the CP peak from background, which simplified the detection of 5 ppb CP in spiked muscle samples. However, where trace levels are concerned, the risk of interfering substances is considerably increased, and the ultimate identification method requires additional specificity.

The method has been applied to the determination of CP residues in chicken tissues. Results of duplicate experiments (Table 2) indicate a rapid decrease in residue levels, especially in liver where a rather high concentration (12 ppm)

Table 1. Recovery of chloramphenicol from spiked calf muscle

Chloramphenicol added, μg/g fresh muscle (ppm)	Rec., %
0.01	78
	77
	81
	78
	78
	69
Mean	76.8
s	4.1
0.025	69
	75
	71
	77
	72
	79
Mean	73.8
s	3.8
0.05	68
	75
	81
	80
	68
	77
Mean	74.8
s	5.7
0.1	77
	79
	73
	73
	69
	79
Mean	75.0
s	4.0

Table 2. Chloramphenicol residues (μg/g fresh tissue) detected in chicken tissues at 4, 6, and 8 h following injection of 20 mg CP/kg

Time after administration, h	Liver	Muscle	Fat
Control	ND[a]	ND	ND
4	12.1	0.04	0.33
	9.6	0.035	0.30
6	0.25	0.03	0.12
	0.18	0.03	0.15
8	0.03	ND	0.10
	0.015	ND	0.05

[a] ND = none detected.

was measured 4 h after administration of the drug. Eight hours after treatment, CP residues were detected and measured at levels below 0.05 ppm. Available methods lack sensitivity to detect residues at such levels.

GC-MS Confirmatory Study

Figure 2 shows the electron impact mass spectrum obtained under our experimental conditions, using standard trimethylsilylated CP. Aside from the 2 major fragments at m/z 225 (base peak) and m/z 208, four much heavier if even less abundant fragments were observed that offered a greater specificity for CP identification. Moreover, the presence of chlorine atoms gives the very characteristic isotope profile of ^{35}Cl and ^{37}Cl for the fragments that possess the dichloroacetate moiety. The choice of the 6 ions took into account these data, to obtain the lowest ionic current background. Moreover, peak areas were measured to establish abundance ratios of the fragments.

Chemical confirmation of the residues that were presumptively identified as CP on the basis of their LC retention time was carried out on tissues of chicken slaughtered after CP administration, when the residual values were the lowest, but still measurable in various tissues. The results are shown in Figure 3. To obtain better accuracy in peak detection, scale factors were computed for every ion monitored so that the highest ionic current, including the background level, reached a value fixed to 2 times the full scale range of the recorder. Scale factor value of 1 corresponded to the maximal amplification of the signal. In spite of the great range of fragment abundance and the various CP peak-to-background ionic current ratios, 30 ng standard CP exhibited 6 well shaped peaks at 2.6 min retention time with low baseline. Similar results were obtained for the tissue sampled 8 h after CP administration, which confirmed that LC peaks detected in liver and fat extracts were due to CP residues. On the contrary, no peak was detected in muscle, but the high background noise level recorded with various ions requires further careful interpretation.

To determine the lowest CP concentration that could be identified in tissues, control chicken muscle samples were spiked with decreasing quantities of standard CP, namely, 21, 14, and 5 ppb, which corresponds to 21, 14, 5, and 2 ng collected, silylated, and analyzed, respectively. No ionic current was recorded for any of the 6 fragments at 2.6 min retention time in control samples. Chicken liver from the 8 h trial, as the spiked chicken muscle samples, exhibited multi-ion responses. However, when CP concentration in the tissues decreased, thereby decreasing the quantity of CP injected into the

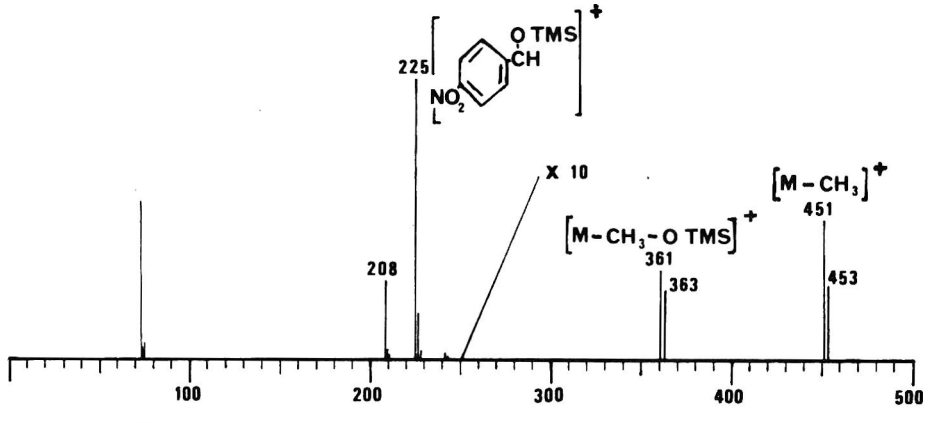

Figure 2. Electron impact mass spectrum of trimethylsilylated CP.

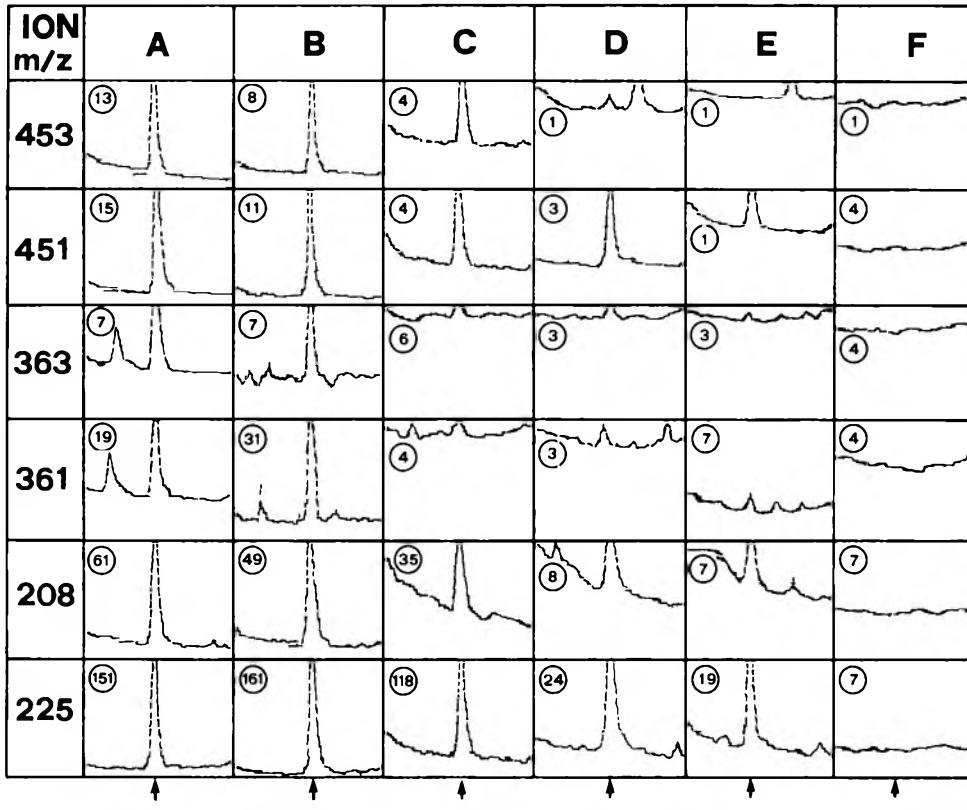

Figure 3. GC-MS SIM recordings of trimethylsilylated standard CP (A) and derivatized LC fractions of control (F), spiked chicken muscle (C, D, E), and liver 8 h after administration (B). Arrows indicate retention time = 2.6 min. Circled numbers are scale factors (see text for explanation).

GC-MS system, the area of peaks of the less abundant ions reached the exclusion threshold set at 10. Fragments m/z 361, 363, and then 453 were rejected successively even when the peaks were still perceptible. However 3 ions, m/z 451, 208, and 225, exhibited measurable responses simultaneously (8 times the threshold value for m/z 451) only for the sample spiked with 5 ppb.

Reliable results were obtained for the intensity ratio of the fragments down to 30 ppb, i.e., about 10 ng equivalent CP injected, under the operating conditions (Table 3). Below this concentration, the precision and reliability of the measurements decreased, due to the sensitivity and stability limitations of the GC-MS system. Thus, a progressive shift of the intensity ratios was observed, even in the fragments with chlorine

Table 3. GC–MS SIM analysis of chloramphenicol residues in fortified and withdrawal tissue samples

Sample	Quantity injected,[a] ng	Replicates	Intensity ratios 225	208	361	363	451	453
Trimethylsilylated standard CP	10	3	100	25.8	3.2	2.4	4.9	2.6
Chicken liver (8 h withdrawal, 34 μg/kg)	12.7	2	100	26.4	2.9	2.3	5.3	3.2
Spiked chicken muscle								
21 μg/kg	7.5	2	100	22.9	—[b]	0.9	3.9	1.6
14 μg/kg	5.5	2	100	19.7	—[b]	—[b]	4.5	1.0
5 μg/kg	1.9	1	100	15.9	—[b]	—[b]	4.5	—[b]

[a] Taking into account yield of overall procedure (75% recovery).
[b] Peak area of fragment below exclusion threshold; intensity ratio not determined.

atoms and only 2 amu apart. At the 5 ppb level, one ratio (m/z 451/225) was still consistent with the value obtained for standard CP.

The requirements generally accepted for confirmatory assay (8) indicate that at least 3 identical ions with constant intensity ratios must be monitored. Down to 15 ppb, these conditions are more than satisfied as 5 and then 4 fragments were monitored. Interpretation at the lowest concentration tested (5 ppb) is rather difficult, because 3 ions are detected but only one ratio is significant. If an unequivocal confirmation of CP cannot be made on the basis of this assessment, a very strong presumption exists.

In conclusion, the methodology described in the paper permits routine control of CP residues in animal tissues at a 10 ppb level, which represents about a 50× increase in sensitivity compared with the available microbiological methods, and confirmatory identification of CP at the 5 ppb level.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Density Meter Determination of Proof of Ethanol-Water Solutions: A Comment

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The official method for density meter determination of proof is lightly biased because the meter is standardized with specific values in vacuum, which is not strictly compatible with the recommended table, that explicity requires "apparent," i.e., "in air" values for entry.

Recently, a method for density meter determination of proof was collaboratively studied (1), and adopted as official first action (2). Although this method might finally free the chemist from cumbersome pycnometry measurements, we point out that the method as described is lightly biased.

An oscillating tube density meter, as is well known, does not actually measure densities, but rather oscillation periods. It has been established by Kratky et al. (3) that a linear relationship exists between the square of the oscillation period and the density in vacuum; the dependence can be mathematically expressed as follows:

$$\rho = (1/A) \times (T^2 - B)$$

where ρ is density in vacuum, T is the oscillation period, and A and B are instrument constants. The linearity was further discussed in a previous paper (4) in connection with the analysis of possible experimental errors. It follows from the rules of mathematics that a linear relationship must also exist between the same values of oscillation period and any other values that are directly proportional to densities in vacuum. Such a linear relationship between densities in vacuum, densities in air, specific gravities in vacuum, and specific gravities in air is well established and documented (e.g., 5, 6, 7). Mathematical expression of 2 of these relationships is given later in this paper.

The value of density or specific gravity of a measured sample is calculated by interpolation of the observed oscillation period for the unknown between 2 values for 2 standards measured initially in a standardization (calibration) procedure. Air and water are generally recommended as the standard media, and the interpolation is finally done automatically by the processor of the instrument. The unit of the result is determined by the units used as the calibration values, densities, or specific gravities of the 2 standards.

To be compatible with AOAC Table 52.003, the method (1) correctly recommends using specific gravities 20°/20° of water and air in calculations of the apparatus constants A and B, viz., 1.000 for water and 0.00120 or 0.00119 for air. While the first is the specific gravity of water (by definition) in vacuum as well as in air, the second applies to specific gravity of air "in vacuum," the specific gravity of air "in air" being necessarily zero. This can be proven by simple common sense—air does not weigh anything in air because the air buoyancy necessarily just cancels out the weight—and also by calculation using the formula relating specific gravity in air (s) to specific gravity in vacuum (d) at 20°/20°:

$$s = 1.00120 \times d - 0.00120, \text{ or}$$

$$d = 0.99880 \times s + 0.00120$$

(Standard values for density of air, 0.0012, and density of weights, 8.000, as recommended by OIML (8) were used in developing the formulas.)

Using values of specific gravities in vacuum for the standardization of the meter, the authors have defined the resulting figures as specific gravities in vacuum, which is not strictly compatible with the recommended table, that explicitly requires "apparent," i.e., "in air" values for entry.

The corresponding bias is rather small: At 0.8000, the difference between in air and in vacuum values is 0.0003, corresponding to 0.03% v/v ethanol; at 0.9000, the difference in specific gravities is 0.0001, corresponding to 0.04% v/v ethanol in this range; close to 1.000, the difference disappears. Because all participants in the

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collaborative study (1) used the same standardization procedure, the bias is not observable in repeatability and reproducibility estimates; it could be observed in the accuracy values, but, because the bias is much smaller than the error on the pycnometry used for comparison (estimated as 0.226 in the study), it is not recognized.

The method (1) provides good and reliable results even with this small bias. For the sake of correctness, we would suggest, however, amending the method in the standardization step so that the result would indicate the desired specific gravity in air. To do so, the value of 0.0000 (zero) should be used in calculating the apparatus constants as follows:

$$A = (T^2_{water} - T^2_{air})/(1.000 - 0.000)$$
$$= (T^2_{water} - T^2_{air}), \text{ and}$$

$$B = T^2_{water} - (A \times 1.000)$$
$$= T^2_{water} - A, \text{ or the equivalent:}$$

$$B = T^2_{air} - (A \times 0.000) = T^2_{air}$$

The calculation would actually be simplified.

The values displayed in the ρ mode should read 1.00000 for water and 0.00000 for the empty, dry tube.

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Improved Cleanup and Derivatization for Gas Chromatographic Determination of Monosodium Glutamate in Foods

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A gas chromatographic procedure is described for determining monosodium glutamate (MSG) in several types of food. A sample is extracted with acetone-water (1 + 1). Acetone is evaporated and an aliquot of the extract is buffered with 1M NH_4OH-1M NH_4Cl pH 9 solution, and chromatographed directly on a column of QAE Sephadex A-25 that has been pretreated with the same buffer. MSG is eluted with 0.1N HCl, and a portion of the eluate is evaporated to dryness and reacted with dimethylformamide(DMF)-dimethylacetal to form the glutamic acid derivative, which is injected into a gas chromatograph and measured by flame ionization detection. Recoveries of MSG from sample fortified at 5-500 mg ranged from 92.8 to 100%.

Monosodium glutamate (MSG) is widely used as a flavor enhancer in food; however, MSG has been implicated as the compound responsible for the Chinese restaurant syndrome (1). In Japan, MSG is registered as a food additive without government regulation. Therefore, labeling MSG content in food is voluntary in the food industry.

Fernandez-Flores et al. (2) used an ion-exchange column for cleanup and isolation of MSG. However, their procedure included an additional step to remove starch and sugars in the extract before ion-exchange column chromatography. Conacher et al. (3) modified the extraction and cleanup steps of their method, and subsequently determined MSG by gas chromatography as the trimethylsilyl derivative. Gal and Schilling (4) determined MSG in food as the *N*-trifluoroacetyl-*n*-butyl ester derivative. These derivatives must be prepared under thoroughly dry conditions because the reagents are moisture-

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sensitive. Therefore, residual water must be removed by freeze-drying or by azeotropic distillation before derivatization.

The present method improves the cleanup and derivatization steps by using QAE-Sephadex as ion-exchange resin and dimethylformamide(DMF)-dimethylacetal as the derivatization reagent. These improvements provide a simple, rapid, and more accurate determination of MSG in foods.

METHOD

Apparatus

(a) *Gas chromatograph.*—Yanaco G-80 (Yanagimoto Mfg Co., Ltd, Kyoto, Japan) equipped with flame ionization detector. Operating conditions: temperatures—injection port 220°C, detector 220°C, column 180°C; nitrogen carrier gas 40 mL/min.

(b) *GC column.*—Glass 2.25 m × 3 mm id, packed with 10% OV-1 on 80–100 mesh Chromosorb WHP.

(c) *High-speed homogenizer.*—Ultra Turrax (Janke and Kunkel, GFR).

(d) *Screw-cap vial.*—With Teflon-lined plastic screw cap (Pierce Chemical Co., Rockford, IL).

(e) *Ion-exchange column.*—25 × 300 mm Pyrex glass with glass stopcocks.

(f) *Hot dry bath.*—With variable temperature control system (±2°C) (Iuchi Seieido Co., Ltd, Osaka, Japan).

(g) *Rotary evaporator.*—Tokyo Rika Co., Ltd, Tokyo, Japan.

Reagents and Materials

(a) *Ion-exchange resin.*—QAE-Sephadex A-25 (Pharmacea Co., Ltd, Sweden).

(b) *Dimethylformamide (DMF)-dimethylacetal.*—Gas chromatographic grade (Wako Pure Chemical Industries Ltd, Osaka, Japan).

(c) *Glutamic acid standard solutions.*—(1) *Stock solution.*—Dissolve 100 mg glutamic acid (Kyowa Hakko Co., Ltd, Tokyo, Japan) in 0.1N HCl in 100 mL volumetric flask and dilute to volume. (2) *Working solutions.*—Dilute aliquots of stock solution with 0.1N HCl to prepare solutions containing 0.02, 0.04, 0.08, 0.16, and 0.32 mg glutamic acid/mL.

(d) *NH_4OH–NH_4Cl buffer solution.*—pH 9.0 (ionic strength 0.1). Add 100 mL HCl to 141 mL 1M NH_4OH and dilute to 1 L with water.

(e) *Samples.*—Seasoning, Kamaboko (fish paste), Chikuwa (fish paste), ham sausage, mayonnaise, and instant noodle soup.

Preparation of Samples

Extract glutamic acid by using acetone–water (1 + 1) as described by Fernandez-Flores et al. (2). For liquid samples, weigh 5 g sample into 200 mL volumetric flask and dilute to volume with acetone–water (1 + 1). For solid samples, weigh 5–10 g chopped sample into 200 mL beaker, add 80 mL acetone–water (1 + 1) and homogenize with high-speed homogenizer for 5 min. Centrifuge 10 min at 3000 rpm, and collect liquid layer. Re-extract residue with 50 mL acetone–water (1 + 1) for 5 min, and centrifuge. Combine liquid layers in 200 mL volumetric flask and dilute to volume with acetone–water (1 + 1).

Ion-Exchange Cleanup

Gradually add 5 g QAE-Sephadex A-25 to 50 mL buffer solution in beaker. Let gel hydrate 5 h. Smoothly pour stirred slurry into chromatographic column to ca 18 cm bed height and convert to Cl^- form by passing 100–200 mL buffer solution through column. Pipet 20 mL portion of 200 mL extract into round-bottom flask, add 0.2 mL 6N HCl, and evaporate extract to dryness on rotary evaporator at 60°C. Quantitatively transfer residue into 20 mL volumetric test tube with 5 mL buffer solution. Rinse flask with two 5 mL portions of buffer solution and add rinse to test tube. Adjust solution to pH 9 with 4N NH_4Cl and dilute to volume with buffer solution. Pipet 2–5 mL aliquot of resulting solution onto column, and after sample solution permeates resin, elute with 0.1N HCl at 2 mL/min. Discard first 25 mL portion of eluate and collect next 30 mL in volumetric tube.

Before further use, wash resin column with 100 mL water and restore to Cl^- form by washing with buffer solution to pH 9.

Derivatization of Sample

Pipet 1 mL aliquot of 30 mL eluate into screw-cap vial and evaporate to dryness on 90°C dry bath under stream of nitrogen. Add 0.3 mL acetonitrile and 0.3 mL DMF-dimethylacetal, tightly close vial with Teflon-lined cap, and shake vigorously. React 30 min on 90°C dry bath, and then cool to room temperature.

Determination

Analyze 5–10 μL aliquot by GC. Calculate monosodium glutamate from following equation:

$$\text{MSG} = \text{glutamic acid} \times 1.15$$

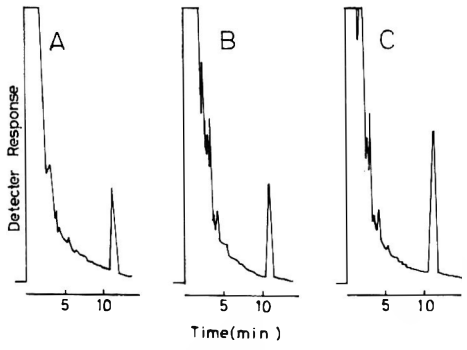

Figure 1. Gas chromatograms of DMF-dimethylacetal glutamic acid derivative: A, standard (0.08 mg); B, obtained from sausage; C, obtained from seasoning.

Preparation of Standard Curve

Pipet 1 mL of working solutions containing 0.02, 0.04, 0.08, 0.16, and 0.32 mg glutamic acid/mL into separate screw-cap vials. Carry out analysis, starting with *Derivatization of Sample.*

Results and Discussion

Extracts of foods containing a relatively large amount of saccharides, such as seasoning and soup, were directly applied onto other resins which have been described in the literature. Recovery of glutamic acid from the resins was variable and was not satisfactory. However, the QAE-Sephadex column gave excellent quantitative recoveries with good precision. This is considered to be due to the reticular structure and higher ion-exchange capacity of the QAE-Sephadex resin. Three HCl solutions, 0.05, 0.1, and 0.2N were studied for their ability to recover glutamic acid from the ion-exchange column. A similar elution pattern was obtained with 0.1 and 0.2N HCl, but the pattern for 0.05N HCl was broad. Therefore, 0.1N HCl was selected, because the QAE-Sephadex was stable during its use. A 30 mL portion of 0.1N HCl was sufficient to yield quantitative recovery of glutamic acid at levels from 0.5 to 20 mg. Other amino acids eluted from the ion-exchange column together with glutamic acid, but no interference was observed on the gas chromatograms.

Quantitative GC determination of glutamic acid has been carried out on the trimethylsilyl and *N*-trifluoroacetyl-*n*-butyl ester derivatives, but these reagents are very sensitive to moisture and require freeze-drying or azeotropic distillation before the derivative procedure. DMF-dimethylacetal has been evaluated principally for the methylation of fatty acids (5), and this reagent has also been used with amino acids in a solution of acetonitrile (6). We used the reagent to derivatize glutamic acid. Optimum derivatization conditions for glutamic acid were heating at 90°C for 30 min with 0.3 mL acetonitrile and 0.3 mL DMF-dimethylacetal. Excellent linearity and reproducibility were obtained over a sample weight range of 0.02–1.0 mg glutamic

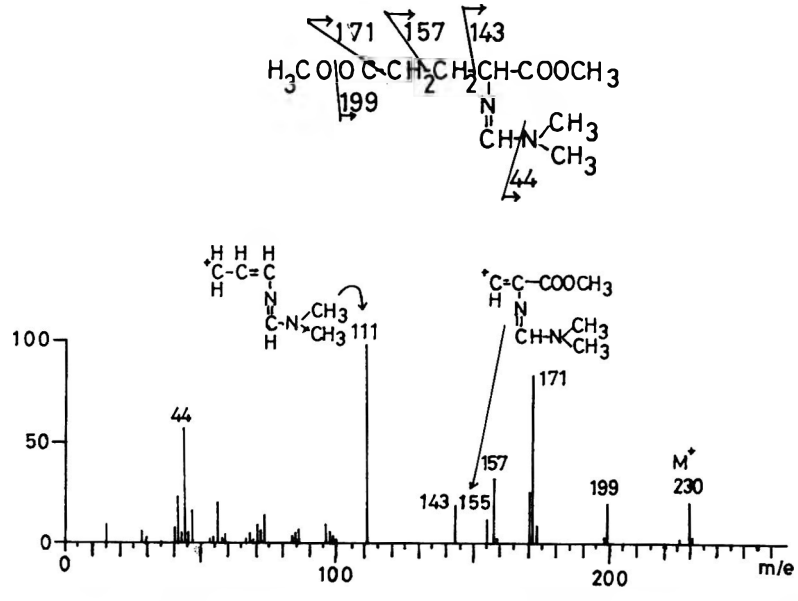

Figure 2. Mass spectrum of DMF-dimethylacetal glutamic acid derivative obtained from seasoning.

Table 1. Recovery of monosodium glutamate (mg) added to foods

MSG present	MSG added	MSG, total found	Rec., %
	Seasoning (Liquid)		
750	500	1217	93.4
635	100	729	94.0
35	50	81.4	92.8
	Kamaboko (Fish Paste)		
160	100	259	99.0
160	100	257	97.0
	Mayonnaise		
9	10	18.5	95.0
9	5	13.7	94.0
	Instant Noodle Soup		
750	150	900	100
750	100	847	97.0
	Average recovery, %		95.8
	Coeff. of var., %		2.7

Table 2. Monosodium glutamate in commercial foods

Food	MSG, %
Seasoning (liquid)	15.0
Seasoning (liquid)	12.7
Seasoning (liquid)	0.87
Seasoning (liquid)	0.70
Seasoning (powder)	19.8
Kamaboko (fish paste)	2.5
Kamaboko (fish paste)	1.6
Kamaboko (fish paste)	0.70
Chikuwa (fish paste)	0.54
Chikuwa (fish paste)	0.45
Ham	1.1
Ham	0.45
Sausage	0.18
Sausage	0.35
Mayonnaise	0.37
Mayonnaise	0.09
Instant noodle soup	7.5
Instant noodle soup	0.73

acid. Furthermore, the derivative was stable for 5 days in a closed vial at room temperature.

A typical chromatogram is shown in Figure 1. The approximate retention time for the glutamic acid derivative was 12 min. The derivative was characterized by GC-mass spectrometry; Figure 2 is a representative mass spectrum of glutamic acid obtained from a seasoning sample.

Recoveries of 5–500 mg MSG added to food samples are shown in Table 1. Average recovery was 95.8% with a range of 92.8–100% and a coefficient of variation of 2.7%.

The present procedure was applied to several commercial foods; MSG content (Table 2) ranged from 0.09 to 19.8%. Of the 5 seasonings, 3 samples contained high levels of MSG (19.8, 15.0 and 12.7%). However, in use, these 3 samples may be diluted about 10 times.

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Gas Chromatographic Determination of Glucono-δ-Lactone in Foods

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A method is described for the gas chromatographic (GC) determination of glucono-δ-lactone in foods. A sample was homogenized with 60-70°C water and filtered. The filtrate was buffered with NH_4OH-NH_4Cl pH 10 solution, and was passed through a QAE-Sephadex A25 column. The column was washed with water and glucono-δ-lactone was eluted with 0.1N HCl. An aliquot of the eluate was evaporated to dryness and derivatized with pyridine, *N,O*-bis(trimethylsilyl)trifluoroacetamide, and trimethylchlorosilane at room temperature. GC separation of glucono-δ-lactone as the TMS derivative was performed on a 2% OV-17 column at 180°C. Recoveries from bread, jelly, soybean curd, and other foods fortified with 0.1% glucono-δ-lactone ranged from 92 to 106%, with standard deviations from 2.2 to 9.8%. The detection limit was approximately 0.025%.

In Japan, glucono-δ-lactone is registered as a food additive and is widely used in soybean curd as a coagulant, in biscuits and bread as an inflating agent, in jelly as an acidulant, and in sausage as pH-lowering agent (1). Glucono-δ-lactone is also used in Europe as a food additive. In West Germany it is used as acidulant in foods, and limit for glucono-δ-lactone in final foods is 1% (10 g glucono-δ-lactone/kg sample); in Sweden, it is used as an acid and maturating agents in foods (2).

Analytical methods for the determination of glucono-δ-lactone are generally based on an enzyme reaction. In Japan, the Ministry of Health and Welfare proposed an enzyme method based on NADPH formation (3).

We have developed an accurate and rapid GC method, and have surveyed contents of glucono-δ-lactone in foods on the market by our proposed method.

METHOD

Reagents

(a) *Glucono-δ-lactone solutions.*—Stock solution: 1000 μg/mL. Dissolve 100.0 mg glucono-δ-lactone analytical grade (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) in 100 mL water. Working solutions: Dilute aliquots of stock solution after adding 2 mL 1N HCl with water to prepare 20 mL solutions containing 25-250 μg glucono-δ-lactone/mL 0.1N HCl.

(b) *NH_4OH-NH_4Cl buffer solution.*—pH 10: Add 100 mL 1N HCl to 250 mL 1M NH_4OH, and dilute to 1 L with water.

(c) *TMS reagents.*—*N,O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) (Nakarai Chemicals Ltd, Kyoto, Japan).

(d) *Anion exchanger.*—QAE-Sephadex A25 (Pharmacia Fine Chemicals).

Apparatus

(a) *High speed homogenizer.*—Ultra-Turrax (Janke and Kunkel KG, GFR).

(b) *Hot dry bath.*—SHD-III (Iuchi Seieido Co., Ltd, Osaka, Japan).

(c) *Gas chromatograph.*—Yanaco G 80 (Yanagimoto Mfg Co., Ltd, Kyoto, Japan) equipped with flame ionization detector. Operating conditions: column temperature 180°C, detector and injection port temperature 225°C; nitrogen flow ca 35 mL/min; glass GC column 225 cm × 3 mm packed with 2% silicone OV-17 on 80-100 mesh Chromosorb W(AW-DMCS).

(d) *Anion exchange column.*—Fill 2 × 30 cm chromatographic column about 1/3 full with buffer solution. Place small plug of glass wool at bottom of column. Stir QAE-Sephadex A25 with buffer solution in beaker. Pour into column to give bed height of 16 cm after settling. Pass 50 mL buffer solution through column.

Procedure

Extraction of glucono-δ-lactone.—Weigh 5 g sample into 300 mL beaker and homogenize with 70 mL water (60-70°C) for 5 min. Pour homogenate into 100 mL cylinder with stopper and dilute to 100 mL with water. Filter supernatant solution through No. 5C paper (Toyo Roshi Co., Ltd, Tokyo, Japan).

Anion exchange cleanup.—Add 30 mL buffer solution to 20 mL filtrate, pour mixture onto QAE-Sephadex A25 column, and elute through column. Wash column with 50 mL water and elute glucono-δ-lactone with 0.1N HCl. Discard first 45 mL of eluate and collect 45-65 mL fraction.

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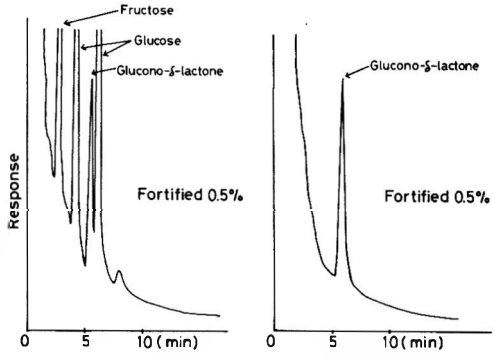

Figure 1. GC chromatograms of glucono-δ-lactone recovered from fortified bread as TMS derivative: left, without cleanup on QAE-Sephadex A25; right, with QAE-Sephadex A25 cleanup.

Trimethylsilylation of glucono-δ-lactone.—Pipet 1 mL eluate into 5 mL test tube with stopper and evaporate to dryness under stream of nitrogen in 80°C dry bath for 30 min. Add 1 mL anhydrous pyridine, 0.1 mL BSTFA, and 0.1 mL TMCS. Shake mixture vigorously and let stand 15 min at room temperature.

GC Determination

Inject 5 μL derivative solution into gas chromatograph and plot peak height (cm) vs glucono-δ-lactone (μg). Detection limit is approximately 0.025%.

Preparation of Standard Curve

Pipet 1 mL of each glucono-δ-lactone standard solution, 25–250 μg/mL, into 5 mL test tube with stopper and carry out analysis in same manner as for sample.

Results and Discussion

Thermostability of Glucono-δ-Lactone in HCl Solution

Solutions of 250 μg glucono-δ-lactone/mL water, 0.02N, 0.05N, and 0.1N HCl were prepared, and 1 mL of each solution was evaporated to dryness under a stream of nitrogen in a 80°C dry bath for 30 min. Each residue was derivatized in the same manner as for samples. No difference in GC peak height between water and any hydrochloric acid solution could be detected. No decomposition of glucono-δ-lactone took place under these conditions, so 0.1N HCl was used to elute glucono-δ-lactone from a QAE-Sephadex A25 column by considering elution time.

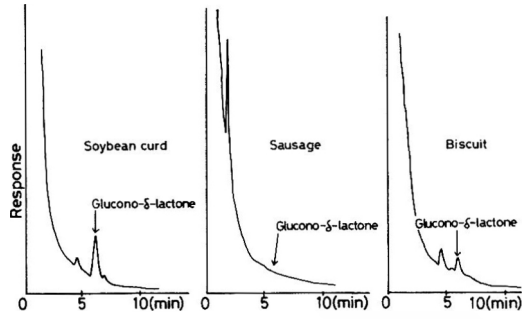

Figure 2. GC chromatograms of glucono-δ-lactone from prepared foods as TMS derivative.

Cleanup of Filtrate

Effective purification of filtrate on the QAE-Sephadex A25 column is shown in Figure 1. GC chromatograms of the trimethylsilyl (TMS) derivative of glucono-δ-lactone recovered from fortified bread show that monosaccharides were eliminated by cleanup on the QAE-Sephadex A25 column.

Interfering Substances

The influence of various substances on cleanup on the QAE-Sephadex A25 column was studied for (*1*) 100 mg glucose and 100 mg fructose, (*2*) 10 mg citric acid, 10 mg malic acid, and 10 mg lactic acid, and (*3*) 10 mg monosodium-L-glutamate. Each of (*1*)–(*3*) was mixed with 5 mg glucono-δ-lactone in 50 mL NH_4OH–NH_4Cl pH 10 buffer solution. Each mixture was passed through a QAE-Sephadex A25 column. Recovery of glucono-δ-lactone mixed with (*1*), (*2*), and (*3*) was 85, 100, and 102%, respectively. Mixture (*1*) had a slight influence on the determination of glucono-δ-lactone.

Recoveries of Glucono-δ-Lactone from Selected Foods

The standard curve for glucono-δ-lactone at 25–250 μg was a straight line. Recovery studies

Table 1. Recovery of glucono-δ-lactone from selected foods

Sample	Added, %	Rec., [a] %	SD
Bread	0.1	94	2.2
	0.5	95	2.9
Jelly	0.1	92	9.8
	0.5	80	2.3
Soybean curd	0.1	106	8.6
	0.5	101	3.6
Sausage	0.1	96	4.7
	0.5	100	3.5

[a] Average of 5 determinations.

Table 2. Determination of glucono-δ-lactone in foods on market

Sample	Found, %[a]
Soybean curd I	0.14
Soybean curd II	0.04
Soybean curd III	ND[b]
Sausage I	ND
Sausage II	ND
Jelly I	ND
Jelly II	ND
Biscuit I	0.04
Biscuit II	ND

[a] Detection limit 0.025%.
[b] ND = none detected.

were carried out by adding 0.1 and 0.5% glucono-δ-lactone to 5 g food samples. Recoveries by the proposed method (Table 1) were 92–106% and 80–101% for 0.1 and 0.5% added glucono-δ-lactone, respectively.

Contents of Glucono-δ-Lactone in Foods on Market

Figure 2 shows typical GC chromatograms of the TMS derivative of glucono-δ-lactone from marketed foods. Results of these analyses are shown in Table 2.

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Pesticide Residue Levels in Foods in the United States from July 1, 1969, to June 30, 1976: Summary

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The U.S. food supply was examined for pesticide residues during the 7-year period covering Fiscal Years (FY) 1970 through 1976. The results, which are contained in the report summarized here, are a continuation of an earlier report for the 6-year period FY 1964-1969.

Scope of Report

The U.S. food supply is routinely monitored by the Federal government for pesticide residues to ensure compliance with regulations governing the safe application of pesticides and to identify patterns of pesticide residue occurrence. These patterns continue to change, but long-term residue patterns may also develop; although use of certain pesticides has been curtailed, their residues are still being detected years later.

Several Federal agencies have related responsibilities in the chemical contaminants area, and pesticide residue data are collected under 3 major Federal programs; 2 of these programs are administered by the Food and Drug Administration (FDA), and the third by the Food Safety and Quality Service of the U.S. Department of Agriculture (USDA).

FDA's Monitoring Program.—FDA uses information on pesticide residue levels in raw agricultural commodities both directly and indirectly to enforce legal tolerances for these chemicals. Findings in domestic surveillance samples in FDA's Monitoring Program assist the agency in predicting the likelihood of finding illegal residues in any specific growing area surveyed. Both domestic and imported commodities are monitored. During FY 1970–1976, FDA's Monitoring Program for raw agricultural commodities examined more than 33 000 domestic and more than 18 000 imported samples that included fresh fruits, fresh vegetables, grains, animal feedstuffs, milk and dairy products, and a variety of processed products. In all, 124 different

[1] Duggan and Associates, Montross, VA 22520.

pesticide chemicals were found in these samples. Residues, mostly at levels lower than 0.5 ppm, were found in more than 56% of the samples, and residues of more than one compound were found in 30% of the samples.

FDA's Total Diet Program.—The Total Diet Program examines ready-to-eat foods to identify problems and trends in dietary intake of pesticide residues. Data from the Total Diet studies are compared with acceptable daily intakes (ADIs) for individual pesticides established by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO). (ADI is the highest daily dosage of a chemical which, during an entire lifetime, appears to be without appreciable risk.) During FY 1970–1976, residues of 44 different chemicals were found in ready-to-eat foods in the analysis of 1980 composites from 165 Total Diet samples. Residue levels in ready-to-eat composites were much lower than in related samples of individual raw agricultural commodities.

USDA's National Residue Program.—The Food Safety and Quality Service samples domestic meat and poultry to detect pesticide residues. Such information is significant because of the tendency of nonpolar organochlorine compounds to accumulate in animal fat and to be magnified in the food chain. For FY 1970–1976, approximately 70% of the more than 15 000 red meat samples examined and approximately 89% of the more than 11 000 poultry samples examined contained residues.

Conclusions

The major findings from all 3 programs are represented by the following conclusions. The incidence of samples of red meat and poultry containing residues was generally greater than for most other food classes. For all food categories, the frequencies and the levels of both organochlorine and organophosphorus pesticide chemicals have been declining; residues of DDT and dieldrin are notable in this respect. The identities of the pesticide chemicals found in raw agricultural commodities are generally the same as those in ready-to-eat foods, although the latter contain much lower levels of pesticide residues. The dietary intakes of pesticide chemicals, as measured by the Total Diet samples, are substantially below the ADIs established by WHO/FAO.

The full report for FY 1970–1976 (approximately 25 pages of text plus 250 pages of indexed tables), which was prepared under contract with the Food and Drug Administration, will not be published in this journal. However, copies may be obtained on request from AOAC, Suite 210, 1111 N. 19th St, Arlington, VA 22209.

FOR YOUR INFORMATION

AOAC to Hold Ninth Annual Spring Workshop and Exposition, April 29-May 2, 1984

The Association of Official Analytical Chemists (AOAC) will sponsor the 9th Annual Spring Workshop and Exposition April 29-May 2, 1984, at the Philadelphia Marriott Hotel, Philadelphia, PA. Technical sessions will provide the latest information on the following subjects: Immunochemistry, Genetic Toxicology, Electroanalytical Techniques, Drugs, Pesticides, Thin Layer Chromatography, Liquid Chromatography Electrochemical Detectors, Gas Chromatography, Toxicology, Nutrient Analysis, Adulteration by Migration of Packaging Material, Drug Metabolism, Environmental Contamination, Liquid Chromatography, Disinfectants, Collaborative Study Procedures, Robotics in Laboratory Automation, Trace Metal Analysis, and Macromolecular Separations.

An exhibition of state-of-the-art scientific equipment will be held in conjunction with this conference.

For information contact: James J. Karr, Pennwalt Technological Center, 900 First Ave, Box C, King of Prussia, PA 19406 (215/337-6560); or Harvey Miller, FDA, US Customhouse, 2nd & Chestnut Sts, Philadelphia, PA 19106 (215/597-4375).

USDA Live Animal Swab Test

The United States Department of Agriculture has developed a new on-farm Live Animal Swab Test (LAST) for antibiotic residues.

As a consumer safety check, LAST can be performed by farmers before livestock are slaughtered and sent to market. LAST helps prevent economic losses caused by residue violations.

A relatively simple and inexpensive microbiological procedure, LAST is performed on urine specimens from cows, calves, and heifers before the animals leave the farm. In the past, residues could be detected only by laboratory tests of carcass tissue. USDA Handbook No. 601, "How to Perform the Live Animal Swab Test for Antibiotic Residues," gives instructions for performing the test and a list of equipment suppliers. An audiotape cassette is also available. To order the handbook or to obtain more information, contact the Food Safety and Inspection Service, USDA, Public Awareness, Rm. 1163-South, Washington, DC 20250; phone: (202) 447-9351.

Meetings

April 29–May 2, 1984: AOAC 9th Annual Spring Training Workshop and Exposition, Philadelphia Marriott Hotel, Philadelphia, PA. For information, see article above.

October 29–Nov. 2, 1984: AOAC 98th Annual International Meeting and Exposition, Shoreham Hotel, Washington, DC.

April 8–11, 1985: AOAC 10th Annual Spring Training Workshop, Downtown Sheraton Hotel, Dallas, TX. Contact: M. Virginia Gibson, FDA, 332 Bryan, Dallas, TX 75204 (214/767-0312); Molly Ready, Alson Labs, 6201 S Freeway, Fort Worth, TX 76134 (817/293-0450).

AOAC Gains New Sustaining Members

AOAC welcomes new additions to the growing list of organizations aware of the need to support an independent methods validation association. A new sustaining member is the Quebec Dept of Agriculture, Sainte-Foy, Quebec, Canada. New private sustaining members are McNeil Consumer Products Co., Fort Washington, PA; Marion Laboratories, Inc., Kansas City, MO; and Stauffer Chemical Co., Richmond, CA.

ISO Standards Published

The following standards have been published by the International Organization for Standardization (ISO), Technical Committee 34—Agricultural Food Products. The standards are available, at prices indicated, from the Food and Drug Administration, Bureau of Foods, HFF-7, 200 C St, SW, Washington, DC 20204.

ISO 6320-1983	Animal and vegetable fats and oils—Determination of refractive index	$ 8.00
ISO 2172-1983	Fruit juice—Determination of soluble content—	$11.00

	Pycnometric method	
ISO 6887-1983	Microbiology—General guidance for the preparation of dilutions for microbiological examination	$11.00
ISO 5530/4-1983	Wheat flour—Physical characteristics of doughs—Part 4: Determination of rheological properties using an alveograph	$15.00
ISO 5223-1983	Test sieves for cereals	$11.00
ISO 6799-1983	Animal and vegetable fats and oils—Determination of composition of the sterol fraction—Method by gas chromatography	$12.00
ISO 6888-1983	Microbiology—General guidance for enumeration of *Staphylococcus aureus*—Colony count technique	$15.00

Courses Offered

Chromatography.—The Varian Instrument Group is offering chromatography courses, ranging from fundamental to advanced topics, periodically through September 1984. All standard courses and most advanced courses are offered at 4 different sites: Walnut Creek, CA; Park Ridge, IL; Sugar Land, TX; and Florham Park, NJ. These courses emphasize hands-on learning and personal instruction, and qualify for college credit and CEUs. Subjects include: fundamentals of gas chromatography (GC); specific detectors, column selection, and capillary techniques in GC; maintenance and troubleshooting of the Model 3700 and 6000 gas chromatographs; gas analysis techniques in GC; fundamentals of liquid chromatography (LC); maintenance and troubleshooting of the Model 5000/5500 liquid chromatographs; VISTA 401/402 chromatography data handling; and toxicology (analytical, clinical, forensic). Advanced course subjects include: advanced LC topics, environmental courses, GC gas analyzers, and data handling. For more information on specific dates, fees, and registration, contact: Varian Instrument Group, 2700 Mitchell Dr, Walnut Creek, CA 94598 (415/939-2400, Ext. 2533).

Journal Begins New Section on Residue Monitoring Data

The Editorial Board and Board of Directors have approved enlarging the scope of the *Journal* to include papers containing tabulations of pesticide and toxic chemical residues in a variety of media, including humans, wildlife, plants, food, air, and water. Government agencies concerned with the control of residues need published credible data to set tolerances and action levels, assure acceptance of commodities in national and international commerce, and obtain acceptance of residue tolerances and standards by international organizations and other countries. These data may also be used to measure the degree of contamination of the environment and in deciding what action to take on specific problems and in specific areas of interest.

To assure the credibility of published residue monitoring data, the protocols for sampling, methods of analysis, quality assurance, and other aspects of specific residue studies will be documented in papers judged acceptable for publication. The objective scientific peer review process of the *Journal* provides assurance that the studies were properly conducted and are clearly described. Publication in the *Journal* assures that the data will be distributed to scientists on a timely basis, will be abstracted by *Chemical Abstracts*, *Biological Abstracts*, *Current contents*, and other secondary services, and will be available to libraries all over the world.

CORRECTIONS

J. Assoc. Off. Anal. Chem. (1981) **64,** 1435–1438, "Protein Nitrogen Unit Precipitation Procedure for Allergenic Extracts: Collaborative Study," by J. C. May and J. T. C. Sih, p. 1436, right column, **"Reagent"**

Change to read:

Phosphotungstic acid (PTA) precipitating solution.—15% PTA in 10% HCl. Dissolve 15.0 g PTA in ca 70 mL H_2O. Add 22.2 mL HCl (sp. gr. 1.19 g/mL, 37.8% HCl) and dil. to 100 mL with H_2O.

p. 1437, left column, **"Determination,"** second to last sentence

Change 0.1N to 0.01N

"Changes in Methods," *J. Assoc. Off. Anal. Chem.* (1982) **65,** 452, column 1, **6.C04,** method title, lines 1 and 2

Change to read:

Pirimicarb (2-(Dimethylamino)-5,6-dimethyl-4-pyrimidinyl Dimethylcarbamate)

"Changes in Methods," *J. Assoc. Off. Anal. Chem.* (1982) **65,** 512, right column, lines 33 and 34; and **66,** 554, right column, lines 24 and 25

Change to read:

2-(Dimethylamino)-5,6-dimethyl-4-pyrimidinyl dimethylcarbamate, . . .

J. Assoc. Off. Anal. Chem. (1983) **66,** 1242–1250, "Flame Photometric Determination of K_2O in Fertilizers: Collaborative Study," by P. F. Kane and R. W. Stringham, p. 1243, left column, **2.D06,** line 19

Change 761 nm to 671 nm

"Changes in Methods," *J. Assoc. Off. Anal. Chem.* (1983) **66,** 513, right column, line 6

Change 761 nm to 671 nm

NEW PUBLICATIONS

Atomic Absorption Spectrometry in Occupational and Environmental Health Practice. Volume 1: Analytical Aspects and Health Significance. Edited by D. L. Tsalev and Z. K. Zaprianov. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1983. Approx. 288 pp., Prepub. Price: U.S. & Can. $78.00, other countries $90.00. ISBN 0-8493-5603-2.

This reference reviews the occupational and environmental health significance of 34 trace elements. It includes information on atomic absorption spectrometry (AAS) research methodology such as sample preparation, interferences, optimization, calibration, and quality control. Volume 1 deals with AAS as an analytical method in occupational and environmental health practice, and includes biological and toxicological characteristics of individual elements. Workers in analytical chemistry, toxicology, medicine, biology, forensic and environmental sciences will find this to be a valuable research tool. It is supplemented with more than 200 tables and illustrations, and outlines over 400 analytical procedures.

The NBS Tables of Chemical Thermodynamic Properties. Selected Values for Inorganic and C_1 and C_2 Organic Substances in SI Units. By D. D. Wagman, W. H. Evans, V. B. Parker, R. H. Schumm, I. Halow, S. M. Bailey, K. L. Churney, and R. L. Nuttall. Available from American Chemical Society, Distribution Office, 1155 Sixteenth St, NW, Washington, DC 20036, 1983. Price: $40.00, outside U.S. $44.00.

Published as Supplement 2 to Volume 11 of the *Journal of Physical and Chemical Reference Data*, this single volume lists 26 000 values for the chemical thermodynamic properties of over 14 000 substances, counting each substance in each phase and each concentration listed for a solution. This comprehensive, updated edition of National Bureau of Standards Technical Note 270 includes standard state data on enthalpy, entropy, Gibbs energy, and heat capacity. Properties of aqueous solutions and pure compounds are also included. All data have been critically evaluated and checked for consistency with thermodynamic constraints by computer programs. These tables are designed to provide scientists with reliable values of chemical thermodynamic properties of the elements and their compounds, useful in such areas as chemical engineering, design, environmental modelling, and chemical research.

Wilson and Wilson's Comprehensive Analytical Chemistry. Volume XV: Methods of Organic Analysis. By L. Mázor. Edited by G. Svehla. Published by Elsevier Scientific Publishing Co., PO Box 211, 1000 AE Amsterdam, The Netherlands, 1983. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017. 530 pp. Price: U.S. & Can. $127.75/Dfl. $300.00. ISBN 0-444-99704-0.

This volume provides a comprehensive survey of all methods useful in the qualitative detection and quantitative determination of organic substances, including medical products. This book contains complete descriptions for the analysis of elements and functional groups, with the more important methods described in sufficient detail to permit implementation in the laboratory. The book can be a useful source of information for chemists, biochemists, pharmacists, teachers of chemistry, physicians, and workers in medical laboratories.

Wilson and Wilson's Comprehensive Analytical Chemistry. Volume XVII: Kinetic Methods of Analytical Chemistry. By M. Kopanica and V. Stará. Application of Computers in Analytical Chemistry. By K. Eckschlager, I. Horsák, Z. Kodejš, Z. Ksandr, M. Matherny, I. Obrusnik, and S. Wičar. Published by Elsevier Scientific Publishing Co., PO Box 211, 1000 AE Amsterdam, The Netherlands, 1983. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017. 446 pp. Price: U.S. & Can. $117.00/Dfl. $250.00. ISBN 0-444-99685-0.

This volume contains 2 contributions: The first deals with the application of kinetic

aspects in analytical chemistry, which involve the determination of traces of metals, analysis of mixtures of chemically related compounds and analysis of biological materials. The respective methods are summarized in tables containing basic data and references. The second contribution includes a survey and examples of the application of computers to the control of analytical experiments and experimental data evaluation. This part includes tables, principal matrix operations and block schemes of computer procedures of regression methods, and it can be used as a guide to analytical data processing.

Standardization and Documentation: An Introduction for Documentalists and Librarians. Published by the International Organization for Standardization (ISO). Available from the American National Standards Institute, Inc., 1430 Broadway, New York, NY 10018, 1983. 94 pp. Price: $17.00. For sales information, or to place orders, call (212)354-3473. For additional information, contact: Walter Gelles, (212)-354-3315.

This newly published manual describes the uses of standards in library work and documentation. It enables information specialists to help users of standards speed their search for the documents and information they need. This manual also provides useful, informative chapters on: standards, national standards bodies of several countries, activities of ISO and the International Electrotechnical Commission, international standards agencies such as the International Organization of Legal Metrology and the Codex Alimentarius Commission, and European standardizing bodies.

Standards and Practices for Instrumentation, 6th Ed. Edited by Lois M. Ferson. Published by Instrument Society of America (ISA), 67 Alexander Dr, PO Box 12277, Research Triangle Park, NC 27709, 1980. ISBN 87664-450-7.

This edition provides complete texts of all current abstracts for over 900 instrumentation-related standards published by ISA and other U.S. and international organizations, and a subject index, cross-referenced to the over 900 titles and abstracts.

Food Research and Data Analysis. Edited by H. Martens and H. Russwurm, Jr. Available from Elsevier Science Publishing Co., Inc., PO Box 1663, Grand Central Station, New York, NY 10163, 1983. 535 pp. Price: $74.00. ISBN 0-85334-206-7.

Containing the proceedings of the International Union of Food Science and Technology Symposium on "Food Research and Data Analysis" held in Oslo, Norway, in September 1982, this book describes computer-aided analysis of multivariate food research data for researchers in food science and technology. The volume explores a range of statistical methods, from "ultra-soft" nonmetric analysis and cluster analysis, through "soft" linear factor and regression models, to "hard" models that require heavy assumptions based on prior knowledge of the data. Practical food applications illustrate the chapters, enabling food researchers with such varying backgrounds as agronomists, microbiologists, chemists, technologists, statisticians, psychologists, and psychophysicists to study the different types of data problems and fully comprehend the applications of the methods.

Along with all invited lectures and poster abstracts presented at the symposium, the book includes a chapter on basic matrix algebra, a simplified overview of multivariate methods, and a bibliography of multivariate data analysis in food research. In addition, 4 keyword index lists are presented, covering food applications and sensory, chemical, and data analysis methods.

Titles in Chemistry and Chemical Engineering, An Academic Press Card Catalog. Available from Academic Press, Inc., a subsidiary of Harcourt Brace Jovanovich, Publishers, 111 5th Ave, New York, NY 10003; (212)741-6800.

This single source of information provides a choice selection of new and recent books and journals from all Academic Press and Grune & Stratton offices around the world. Features of this new card catalog include: standard 3 × 5 in. size, complete bibliographic and narrative description, quick referencing, sorting by subject, and ease of ordering and control.

Nutritional Bioavailability of Zinc. Edited by George E. Inglett. Available from

American Chemical Society, Distribution Office Dept 240, 1155 16th St, NW, Washington, DC 20036, 1983. 280 pp. Price: U.S. & Can. $35.95, other countries $43.95. ISBN 0-8412-0760-7.

This book is based on a symposium sponsored by the Division of Agricultural and Food Chemistry of the American Chemical Society, series No. 210. The volume offers current perspectives and future directions in zinc research. Some topics of interest include: experimental zinc deficiency in humans: an overview of original studies; trends in levels of zinc in the U.S. food supply, 1909–1981; zinc absorption in humans: effect of age, sex, and food; and influence of dietary fiber, ascorbic acid, and past dietary practices.

Electrophoretic Techniques. Edited by C. F. Simpson and M. Whittaker. Published by Academic Press Inc. (London) Ltd, 24-28 Oval Rd, London NW1 7DX, England, or Freepost, London NW1 1YA. Also available from Academic Press, Inc., 111 5th Ave, New York, NY 10003, 1983. 290 pp. Price: U.S. $44.00/£26.00 (UK only). ISBN 0-12-644480-3.

This volume deals with both analytical and preparative electrophoretic separations. It begins with a review of the basic forces which control electrophoresis separations, followed by a consideration of the applications of a variety of techniques to the separations of proteins. Reviews of isoelectric focusing, and its reference to agarose gels and cellulose acetate membranes are included, together with a chapter giving precise methodologies in the new technique of ultra-thin layer isoelectric focusing. Other topics covered include gel immunoelectrophoresis and analytical isotachophoresis, and a discussion of the equipment necessary for extending sample size. This volume provides both theoretical and practical expertise which enables research workers and postgraduates in chemistry, biochemistry, and other life sciences to use these new techniques to the full.

New Frontiers in Food Microstructure. Edited by D. B. Bechtel. Published by the American Association of Cereal Chemists (AACC), 3340 Pilot Knob Rd, St. Paul, MN 55121, 1983. 400 pp. Price: $44.00 AACC members; $48.00 nonmembers. ISBN 0-913250-32-5. For more information, contact R. J. Tarleton, (612)454-7250.

The book is based on papers presented at a symposium in San Antonio, TX, during the AACC 67th Annual Meeting. It covers a variety of microscopic procedures used in the most basic to the most elaborate laboratories. Chapters review a range of techniques and equipment from "simple" free-hand sectioning to ultramicrotomy; from dissecting microscopes to high-voltage electron microscopes; from simple observations to quantitative image analysis; and from general staining to sophisticated cytochemical and elemental analyses. It emphasizes how techniques are conducted. It also discusses where additional in-depth information on microscopic procedures can be found.

AUTHOR INDEX

SUBJECT INDEX

Custom packing HPLC columns has become our specialty. Any length, several ID's (including 3.2mm) and almost any commercially available packing material may be specified. We'll supply the column others won't.

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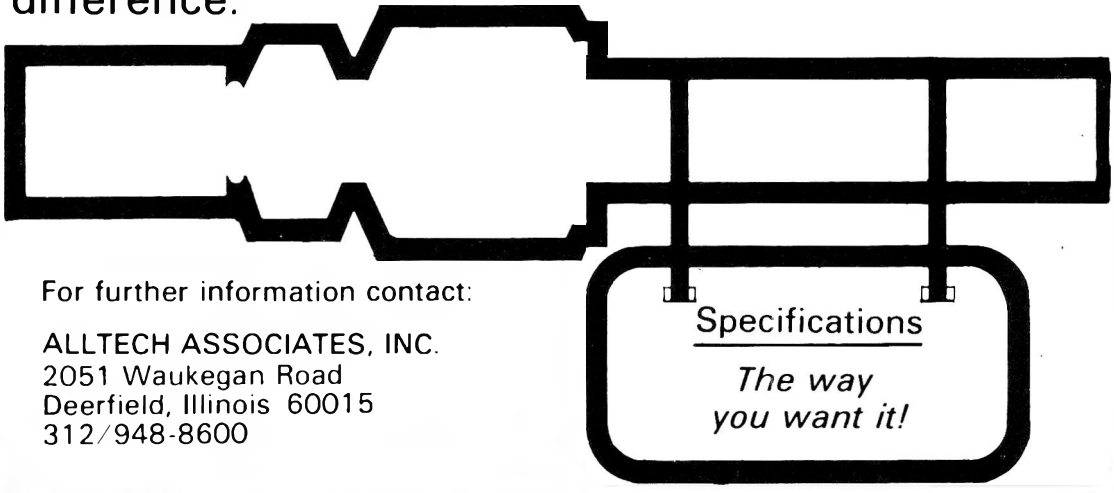

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