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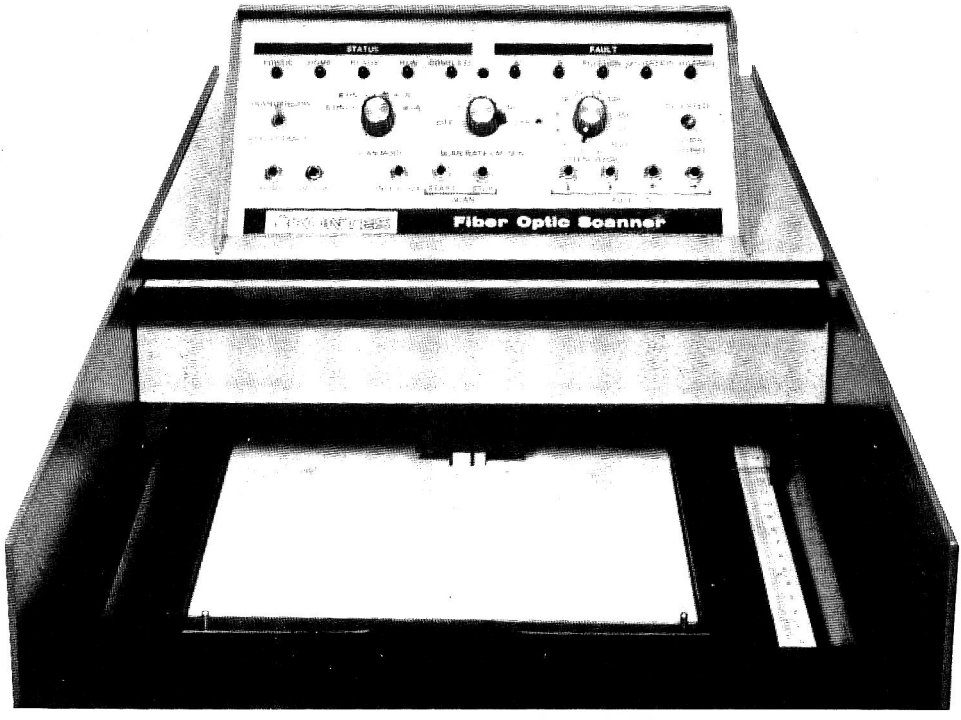

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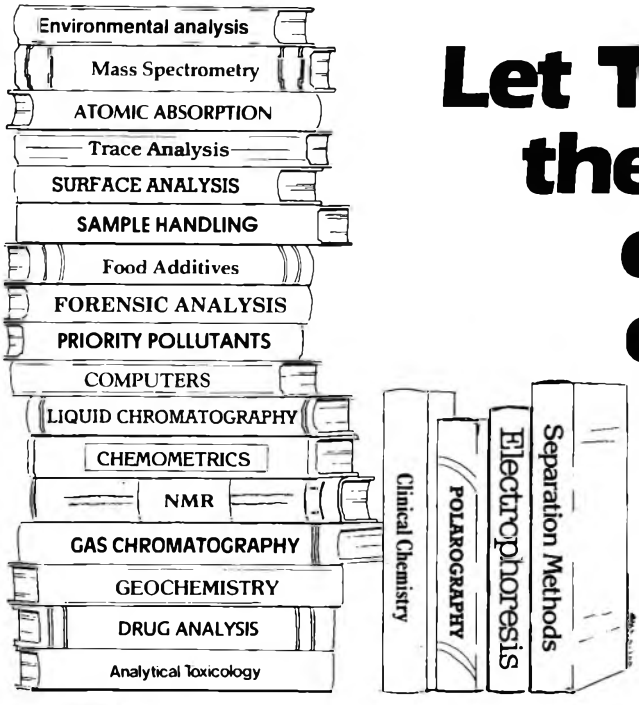

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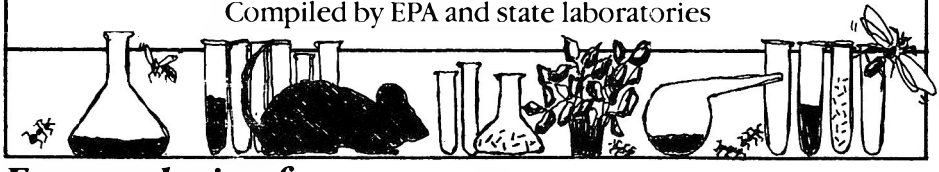

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Related to Agriculture and the Public Health

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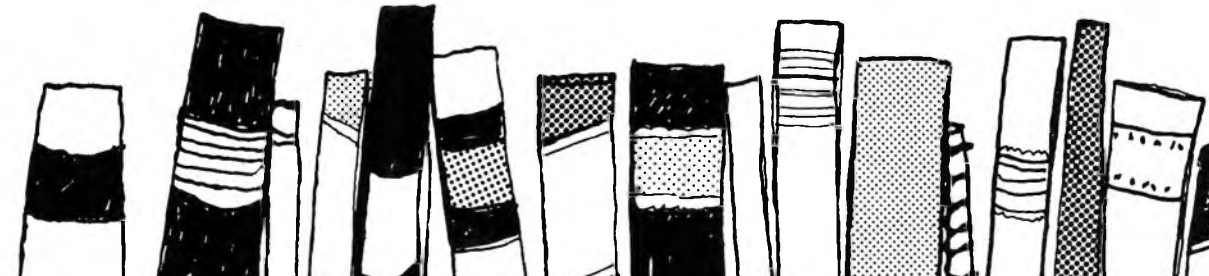

MYCOTOXINS

Rapid Thin Layer Chromatographic Determination of Zearalenone in Corn, Sorghum, and Wheat

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A rapid method is described for determining zearalenone in corn, sorghum, and wheat. The mycotoxin is extracted with a mixture of acetonitrile and 4% KCl in HCl. The extract is cleaned up with isooctane, evaporated, and redissolved in chloroform. Zearalenone is separated by thin layer chromatography; identity is confirmed with various developing solvents and spray reagents. Zearalenone is then quantitated by the limit detection method. The minimum detectable concentration is 140-160 μg/kg when aluminum chloride solution is used as spray reagent, and 85-110 μg/kg when Fast Violet B salt is used as spray reagent.

Zearalenone, or F-2 toxin, is an estrogenic metabolite produced by *Fusarium roseum* and to a lesser extent by several other *Fusarium* species (1–5). *Fusarium roseum* can produce high quantities of zearalenone (3000–15 000 ppm) (5). *Fusarium moniliforme* synthesizes low quantities of this mycotoxin (1–19 ppm) (5). *Fusarium roseum* shows maximum growth at 24–27°C, and zearalenone is produced at 12–14°C (3). Nevertheless, strains of *Fusarium roseum* 'Gibbosum' and *Fusarium roseum* 'Semitectum' were isolated from blighted sorghum grain that produced higher yields at 25°C than at 10°C when grown on autoclaved sorghum (6). Infection and zearalenone production in corn may occur in the field, but it is probably more important in stored corn when temperatures are 6–18°C and moisture content is 23% or greater (7).

Zearalenone has been found in maize, corn screenings, wheat, sorghum, barley, oats, sesame seed, hay silage, and various mixed feeds at concentrations between 0.1 and 2909 ppm (1, 3, 4, 8, 9, 10). Eppley et al. (11) reported incidences of zearalenone in marketable corn at concentrations between 0.1 and 0.5 ppm. The occurrence of zearalenone has been summarized by Stoloff (12), who reported concentrations between 450 and 800 μg/kg in corn. Schroeder and Hein (6) reported the isolation of zearalenone from freshly harvested grain sorghum, and Marasas et al. (13) found zearalenone in corn at concentrations from 2.4 to 12.8 ppm.

The estrogenic syndrome in swine, poultry, and dairy cattle is well documented (1, 9, 14), along with one case of zearalenone mycotoxicosis affecting horses (10). Good methods are available for determining zearalenone by TLC (15–25). However, none of these procedures is rapid (i.e., 15, 16, 23, 25). Some methods (15–17, 20, 24, 25) have good sensitivity for zearalenone (10–50 μg/kg) when TLC is used, depending on the method and the product analyzed. When liquid chromatography is used, the sensitivity is better, 5 μg/kg (17) and 1 μg/kg (24).

The following method is less sensitive than methods previously mentioned, but physiologically significant zearalenone concentrations are higher than the limits of detection (1, 4). Normally, concentrations between 1 and 5 ppm zearalenone are significant, based on responses obtained in test animals (1). The following is derived from the method we have used routinely for some years in our laboratory (21, 22) with a series of improvements (26, 27). If only zearalenone in corn, sorghum, and wheat is analyzed, it is possible to process 8 samples in 2.5–3 h for qualitative analysis and an additional hour for quantitative analysis. The method has been tested for determining zearalenone in barley and oats with good results.

METHOD

Apparatus

(a) *Shaker (wrist or rocker-type).*—Bicasa K30/300 BE38 adaptable to 500 mL Erlenmeyer flask, or equivalent (Garberi Products, Barcelona, Spain).

(b) *Glass flask.*—Amber, pear-shape flask, 250

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mL (Afora No. 5922, Afora Products, Barcelona, Spain). One end narrows to 2 cm × 1 cm id. Narrow section is graduated as accurately as possible to ca 1.5 mL capacity with marked 0.1 mL divisions. Other end of flask has 29/32 ground-glass neck, adaptable to rotary evaporator and glass stopper. Glass must be strong enough to withstand vacuum.

(c) *Multiple evaporation system.*—Buchi No. RSB/40-5-100, or equivalent adaptable to rotary evaporator and to pear-shape glass flask (Buchi Products, Switzerland).

(d) *Membrane vacuum pump.*—KNF N-035 ANE with Teflon valves and membrane, or equivalent (Garberi Products).

(e) *TLC plate scriber.*—Applied Science Laboratories No. 17102, or equivalent.

(f) *Hypodermic syringe.*—50 mL; needle should consist of 8–9 cm long Teflon tube, 1–2 mm id.

(g) *Chromatographic separating chambers.*—Desaga No. 120167, 210 × 88 × 210 mm, or equivalent.

(h) *Ultraviolet lamp.*—Camag standard 29200 or 29230 with blue-violet filter (UG-5, Schott, Mainz, GFR) passing 366 and 254 nm UV light. Place TLC plates on black background 10 cm from lamp. Carry out observations in completely dark room. Protect eyes with yellow filter.

(i) *Yellow filter.*—GG-4 (Schott), or equivalent.

(j) *TLC plates.*—20 × 20 cm glass, precoated with 0.25 mm Sil G-25HR (Macherey-Nagel and Co., Duren, West Germany, No. 809033). Use TLC plate scriber to divide plates into twenty 1 cm strips. Activate plates by heating 30 min at 110°C. Store in desiccating storage cabinet. Use plates at room temperature.

(k) *Tube shaker.*—Mixo-Tube 30, or equivalent.

(l) *Other apparatus.*—As described in Refs 22, 27.

Reagents

(a) *Aluminum chloride solution.*—Dissolve 20 g reagent grade $AlCl_3 \cdot 6H_2O$ in 100 mL ethanol.

(b) *Fast Violet B salt solution.*—Dissolve 0.7 g Fast Violet B salt SIGMA F 4377 or 1631 in 100 mL water. Store in refrigerator for maximum of 5 days.

(c) *Buffer solution.*—pH 9.0. Mix 50 mL 0.025M sodium borate and 4.6 mL 0.1M HCl.

(d) *Mycotoxin standard.*—Zearalenone (Sigma Chemical Co. No. Z-5125). Store in well sealed, aluminum foil-wrapped containers at 4°C.

(e) *Mycotoxin standard solution for qualitative analysis (internal and external standard).*—Zearalenone, 65 μg/mL. Prepare with reagent grade $CHCl_3$. Store solution in well sealed, aluminum foil-wrapped containers or in amber vials at 4°C. Solution should be room temperature when used. Check standard as in Ref. 15.

(f) *Mycotoxin standard solution for test sensitivity (detection limit, μg/spot) on TLC plate.*—Prepare fresh with reagent grade $CHCl_3$: 5 μg zearalenone/mL (test with $AlCl_3$ spray solution) and 2 μg zearalenone/mL (test with Fast Violet B salt/pH 9.0 buffer/50% H_2SO_4 spray solutions).

(g) *Developing solvents for TLC.*—Prepare fresh with reagent grade as shown. (Caution: Chloroform is a possible carcinogen.)

1. Toluene–ethyl acetate–$CHCl_3$ (95 + 55 + 50)
2. Toluene-ethyl acetate-$CHCl_3$-90% formic acid (100 + 50 + 50 + 0.5) (26)
3. Toluene-$CHCl_3$-acetone (30 + 150 + 20) (21, 22)
4. Toluene-$CHCl_3$-acetone-90% formic acid (30 + 150 + 20 + 0.5) (26)
5. Benzene-$CHCl_3$-acetone (85 + 85 + 30)
6. Toluene-ethyl acetate-90% formic acid (100 + 95 + 5) (27)
7. $CHCl_3$-acetone (176 + 24) (21, 22)
8. Benzene-acetic acid (180 + 20) (19)
9. Ethyl ether-hexane (120 + 80) or (110 + 90) (27)

Extraction and Cleanup

Weigh 60 g sample, ground fine enough to pass 0.8–1 mm mesh sieve, and place in 500 mL amber Erlenmeyer flask with glass stopper. Add 180 mL acetonitrile and 20 mL solution of 4% KCl in 0.1N HCl, stopper (secure stopper with masking tape), and shake vigorously 30 min. Filter extract through 9 cm MN 640w paper. Collect 60 mL filtrate and place in 150–200 mL amber separatory funnel (PTFE stopper and stopcock). Add 70 mL isooctane, stopper, and shake 20 s. Let separate into layers and remove top layer of isooctane with hypodermic syringe, taking care not to remove acetonitrile lower layer (it does not matter if a small amount of isooctane remains) (21, 22). Repeat previous operation twice with 60 mL isooctane each time, removing isooctane after each washing as mentioned above.

Filter lower layer of acetonitrile through 9 cm MN 640w paper filled with anhydrous sodium sulfate. Collect filtrate in pear-shape glass flask. Add 10 mL $CHCl_3$ to separatory funnel, rinse, and filter rinse through same sodium sulfate and

add to filtrate already collected. Wash anhydrous sodium sulfate with three 10 mL portions of $CHCl_3$, adding filtrate to that already collected. Evaporate in rotary evaporator to 0.5 mL at 50–55°C with vacuum (depression = 0.6–0.85 kg/sq. cm). Cool, and wash walls of glass flask with two 1 mL portions of $CHCl_3$, carrying liquid to narrow graduated section. Evaporate to 0.1–0.2 mL as done previously. (When vacuum is interrupted, nitrogen gas must be added.) Cool, and add $CHCl_3$ to 1 mL division of narrow graduated section. Stopper and shake on tube shaker. Final extract volume for TLC will be 1.0 mL (1.0 mL $CHCl_3$ solution = 18 g extracted sample). Transfer final extract to amber minivial with Teflon stopper. Stopper, and let stand 5–10 min.

Thin Layer Chromatography

Prepare 3 plates, A, B, and C. On imaginary line 4 cm from bottom, place two 10 μL spots of extract solution sample, at 1 cm intervals, in middle of strip (do not use strips at edges of plate). To one 10 μL spot, superimpose 5 μL standard solution of zearalenone (internal standard). On a clear strip, spot 5 μL standard solution of zearalenone (external standard). Draw transverse line on TLC plates 11–12 cm from imaginary spotting line as solvent stop.

In TLC separating chamber, place ca 200 mL developing solvent, which should reach a height of ca 1.4–1.5 cm; cover and let stand 5 min. Then insert TLC plate in vertical position with silica gel surface 3–5 cm from front wall of developing chamber. Cover and develop in unsaturated chamber until solvent front reaches indicated height.

Develop plate A with developing solvent 1 or 3, and confirm with plates B and C by developing with solvents 7 and 9, respectively. Remove plates from tanks and let plate A dry in dark hood. Force final drying if necessary with gentle current of nitrogen gas. Air-dry plates B and C 5–10 min only in dark hood (do not overdry).

Observe plates under 366 and 254 nm UV light. Under 366 nm, zearalenone shows very faint blue spot. Under 254 nm, zearalenone shows bright blue spot (this is first confirmation (18, 19).

Spray plate A with aluminum chloride solution and observe under 366 and 254 nm UV light; zearalenone shows bright blue spot fluorescence, especially under 366 nm UV light. Heat 10 min at 105°C (28), cool, and spray again with aluminum chloride solution (not heated) (21, 22) and observe under 366 and 254 nm UV light: zearalenone shows bright blue-violet fluorescence spot with improved visualization with respect to background after this second spraying (21, 22).

Table 1. Sensitivity (detection limit, μg/spot) on TLC plate for zearalenone with pure standard solution and with standard toxin added to 10 μL extract of various products

Visualization (see text)	Soln of std, μg	Std toxin added to 10 μL product,[a] μg
254 nm UV	0.02	0.03
$AlCl_3$/UV 366, 254 nm	0.02	0.02
Fast Violet B salt / pH 9.0 buffer / 50% H_2SO_4 (visible)	0.008	0.008–0.01

[a] Sample extract solution of the following products: corn, sorghum, wheat.

Spray plates B and C with 0.7% solution of Fast Violet B salt (17) (spray evenly). Then spray with pH 9.0 buffer solution until silica gel layer appears wet. Dry in air current ca 5–10 min and observe under visible light; zearalenone = pink spots. Then spray TLC plates with 50% H_2SO_4 and heat 5 min at 120°C (17) and observe under visible light; zearalenone shows mauve spots.

Other developing solvents for zearalenone: Nos. 2, 4, 5, 6, 8. For aluminum chloride spray detection, developing solvents 1 to 9 are satisfactory. For Fast Violet B salt spray detection, developing solvents 1, 3, 5, 7, 8, 9 are satisfactory. Compare sample chromatogram patterns with those for internal standard and external standard (same R_f, and color identical to zearalenone).

Quantitative Analysis

Proceed to quantitative analysis where qualitative analysis is positive. Spot series of dilutions from sample extraction solution to determine lowest dilution at which mycotoxin can still be detected. From lower limit of detecting standard (sensitivity on TLC plate, μg/spot) and dilution factor, calculate quantity of mycotoxin (21, 22).

Applying same conditions used for qualitative analysis, relate volume of least spot for which mycotoxin can be verified with sensitivity on TLC plate for mycotoxin in question (see Table 1).

$$\mu g \text{ zearalenone/kg sample} = (L \times F \times 10^6)/(a \times W)$$

where L = sensitivity on TLC plate (see Table 1),

Table 2. Recovery (%) [a] of zearalenone added to various products

	Corn					Sorghum					Wheat				
	Added, μg/kg					Added, μg/kg					Added, μg/kg				
	200	350	500	750	1000	200	350	500	750	1000	200	350	500	750	1000
	96	99	97	99	99	93	95	95	99	98	96	97	98	99	100
	98	98	98	99	98	94	97	95	98	97	95	98	97	96	98
	97	98	97	100	98	94	96	97	98	98	93	98	96	98	99
	97	97	98	98	98	95	96	98	97	98	93	96	97	98	98
	—	98	—	—	—	—	96	—	—	—	—	96	—	—	—
Mean	97	98	97	99	98	94	96	96	98	98	94	97	97	98	99

[a] Based on 5 determinations for 350 μg/kg of zearalenone added and 4 determinations for 200, 500, 750 and 1000 μg/kg of zearalenone added.

μg/spot of corresponding mycotoxin; F = total factor of dilution in mL; a = volume, μL, corresponding to least spot of mycotoxin detectable on TLC plates; W = weight of sample, g (see text = 60 g)

Results and Discussion

Table 1 gives the sensitivity on a TLC plate (limit of detection, μg/spot) of zearalenone in pure solution and in 10 μL extracts of various products after development.

Table 2 gives recovery (%) of zearalenone, based on 5 determinations for 350 μg/kg of zearalenone, and 4 determinations for 200, 500, 750, and 1000 μg/kg of zearalenone added. Minimum detectable concentrations are 140–160 μg/kg for aluminum chloride spray and 85–110 μg/kg for Fast Violet B salt spray.

In analyzing for zearalenone, care must be taken that diameter of spot for total volume deposid does not exceed 2–2.5 mm (dry spots with nitrogen gas). Both natural and artificial direct light should be avoided during analysis, and working temperature should be 22–24°C. All analytical steps should be completed promptly, especially quantitation by the detection limit method; mycotoxin quantities are low and losses can occur.

By using the method described here, we found zearalenone in 5 samples of marketable sorghum (1500–4500 μg/kg), although mycological analysis of these samples did not show *Fusarium* spp. These 5 samples were also analyzed by other TLC methods (15, 21, 22) and the results were similar. A maize determined to be contaminated with 72 ppm zearalenone by previous methods (21, 22, 15) and the method described here, showed 970 000 col/g of *Fusarium* spp. in mycological analysis; 85% were *Fusarium moniliforme* and 15% were *Fusarium roseum*. Three samples of maize and one sample of sorghum were contaminated with zearalenone in concentrations of 620, 870, 1100, and 760 μg/kg, respectively, according to previous methods (15, 21, 22); analysis of the same samples by the method described here showed similar results. Three samples of maize and 2 samples of wheat were contaminated with zearalenone at 900, 1200, 2000, 2500, and 3000 μg/kg, respectively (15, 22, 27); analysis of these samples with the present method showed similar results. Mycological analysis showed *Fusarium roseum* and *Fusarium moniliforme* in the maize, but no *Fusarium* spp. in the wheat. The 2 previous samples of wheat were also analyzed by the reaction with bisdiazotized benzidine (29).

The presence of zearalenone but the absence of *Fusarium* spp. is not as unusual as it would appear. Taking into account the mode of invasion and the production of zearalenone by *Fusarium* spp. growing on maize or sorghum stored after harvest but before drying (1), it is possible that the drying process killed the fungi but did not degrade the mycotoxin.

We analyzed some samples of barley and oats with good performance and without interferences, but have not done a sufficient number of routine analyses.

Analysis for zearalenone in sorghum may reveal a visual fluorescence (possibly alternariol methyl ether (17)) with characteristics similar to zearalenone and appearing at nearly the same R_f as zearalenone. With developing solvents $CHCl_3$–acetone (176 + 24) and ethyl ether–hexane (120 + 80) or (110 + 90) and under the conditions described here, this interference is separated somewhat compared with internal standard zearalenone superimposed on the sample extract solution. To avoid confusion, the reac-

tion with Fast Violet B salt (17) may be applied; alternariol methyl ether does not react with this diazonium salt (17).

Quantitation by the limit detection method is more accurate than visual comparison with different intensities of standard (15). It is easier for the human eye to determine the least spot of mycotoxin detected in a contaminated sample than to compare different intensities of standard. Quantitation by serial dilution is time consuming, but with practice can be less labor-intensive.

Quantitation by instrumental analysis (densitometer) improves the sensitivity of the TLC plate. The wavelength of emission is about 390 nm and optimum excitation wavelength occurs at about 310 nm for zearalenone. UV detection is not possible because the wavelength of irradiation is 366 or 254 nm. The sensitivity (limit of detection, μg/spot) on TLC plates for zearalenone by densitometry is about 0.003–0.004 μg/spot, and the minimum detectable concentration (μg/kg) should be about 25–30 μg/kg.

We have determined that extraction of mycotoxin with acetonitrile and 4% KCl in HCl (9 + 1) gives better recoveries of mycotoxin and cleaner extracts than extraction with acetonitrile in aqueous 4% KCl (9 + 1).

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High Pressure Liquid Chromatographic Determination of an *O*-Methyl,Methyl Ester Derivative of Ochratoxin A

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The mycotoxin ochratoxin A (OA) was derivatized to an *O*-methyl,methyl ester (Me_2) with diazomethane and then determined by high pressure liquid chromatography (HPLC). Both OA and OA-Me_2 were chromatographed by reverse phase HPLC with a mobile phase of acetonitrile-water (60 + 40). An increase in retention time of 309 s was observed with OA-Me_2 which was detectable at 254 nm at levels as low as 3 ng. Recovery of OA as OA-Me_2 from chicken kidney homogenates and human plasma was quantitative following simple extraction and cleanup procedures, reaction with diazomethane, and HPLC analysis. The novel method described should prove useful for measuring and confirming OA in tissues and in further studies on the biological fate of this mycotoxin.

The mycotoxin ochratoxin A (OA), a dihydroisocoumarin derivative (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin) linked to L-β-phenylalanine through the 7-carboxy group, is a natural contaminant of foods and feeds (1-6). Numerous toxicological responses to OA have been reported, including teratogenicity in the mouse (7, 8), rat (9-11), hamster (12), and chick embryo (13, 14); cytotoxicity in cultured monkey renal epithelial cells (15, 16); and iron deficiency and bone and intestinal abnormalities in chickens (17-19). OA alters renal function in a manner suggestive of nephrotoxicity (20), and this mycotoxin has been linked directly as a causative factor in mold-induced nephropathy in pigs in Denmark (21). In addition, OA is believed to be involved in the human disease endemic Balkan nephropathy, a frequently fatal chronic renal disorder afflicting rural populations in Bulgaria, Romania, and Yugoslavia (21, 22).

Procedures for the quantitation of OA in foods, feeds, and animal tissues include thin layer chromatography (TLC) (23), minicolumn techniques (24, 25), immunofluorescence microscopy (26), a spectrophotometric method based on enzymatic cleavage of OA to ochratoxin-α (a common urinary metabolite) and phenylalanine (27), an enzyme-linked immunosorbent assay (28), and numerous methods using high pressure liquid chromatography (HPLC) (29-33). We recently demonstrated that methylation of penicillic acid results in a product with shifted retention time on HPLC, which represents a property useful in chromatographic resolution from co-eluting contaminants and in confirmation of this mycotoxin (34). The current report describes a similar method for derivatization of OA with diazomethane and quantitation by HPLC.

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METHOD

Apparatus

(a) *Liquid chromatograph.*—Waters Model ALC-204 (Waters Associates, Inc., Milford, MA) equipped with Model 440 UV detector with 254 nm primary filter, Tracor 970 A variable wavelength UV detector, U6K septumless injector, M-6000 and M-45 pumps, and RCM-100 radial compression module.

(b) *HPLC column.*—Waters Radial-PAK A octadecylsilane (10 μm particle size). Operating conditions: chart speed 0.5 in./min; flow rate 2.0 mL/min; sensitivity 0.005 AUFS; temperature, ambient; injection volumes, 10 μL.

(c) *HPLC recorder.*—Houston Instrument Series B-5000 Omni Scribe (Austin, TX).

(d) *Integrator.*—Columbia Scientific Industries Model CSI 38 (Austin, TX).

(e) *Spectrophotometer.*—Cary Model 219 UV-visible recording spectrophotometer (Varian Associates, Palo Alto, CA).

(f) *Thin layer chromatographic (TLC) apparatus.*—Brinkmann Silplate F-22, 0.25 mm gel thickness, with fluorescent indicator (Brinkmann, Westburg, NY). Develop in ether-hexane (2 + 1) solvent system. Visualize compounds under shortwave UV light.

(g) *Mass spectrometric (MS) apparatus.*—Varian/MAT Ch-7 mass spectrometer with 620L Varian data system. Spectra were recorded by direct insert probe analysis at 70 eV.

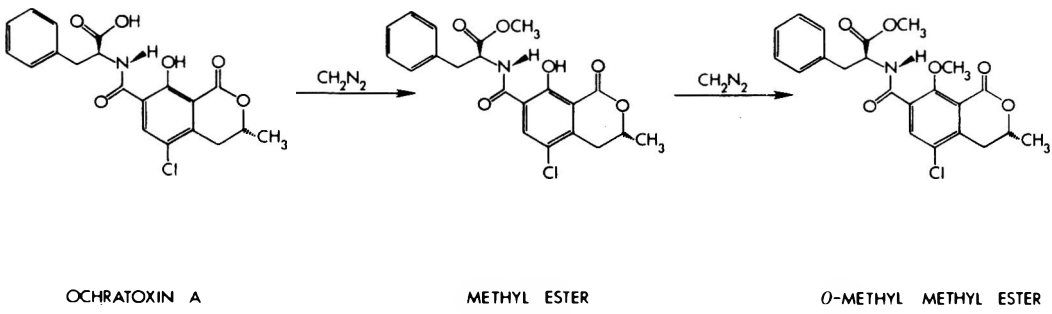

Figure 1. Structure of ochratoxin A and reaction products with diazomethane.

(h) *Fourier transform nuclear magnetic resonance spectroscopy (FT-NMR) apparatus.*—JEOL FX-900, spectra recorded in deuterochloroform, chemical shift reported as parts per million downfield from tetramethylsilane.

(i) *Refrigerated centrifuge.*—Beckman Model J2-21 and Type JA-20 aluminum fixed-angle rotor (Palo Alto, CA).

Reagents

(a) *Solvents.*—Acetonitrile, acetone, chloroform, ethyl acetate (Burdick & Jackson, Muskegon, MI); demineralized and triple-distilled water.

(b) *HPLC mobile phase.*—Acetonitrile–water (60 + 40).

(c) *Ochratoxin A.*—Makor Chemicals Ltd, Jerusalem, Israel. Purity (99–100%) estimated by TLC, HPLC, and MS.

(d) *Diazomethane.*—Prepare ethereal ethanolic solution of diazomethane from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co., Milwaukee, WI). Store at −80°C prior to immediate use.

(e) *Ochratoxin A standard solution.*—0.5 mg/mL in acetonitrile. Store in dark at −30°C. Further dilutions made in acetonitrile as required.

(f) *O-Methylochratoxin A, methyl ester derivative*

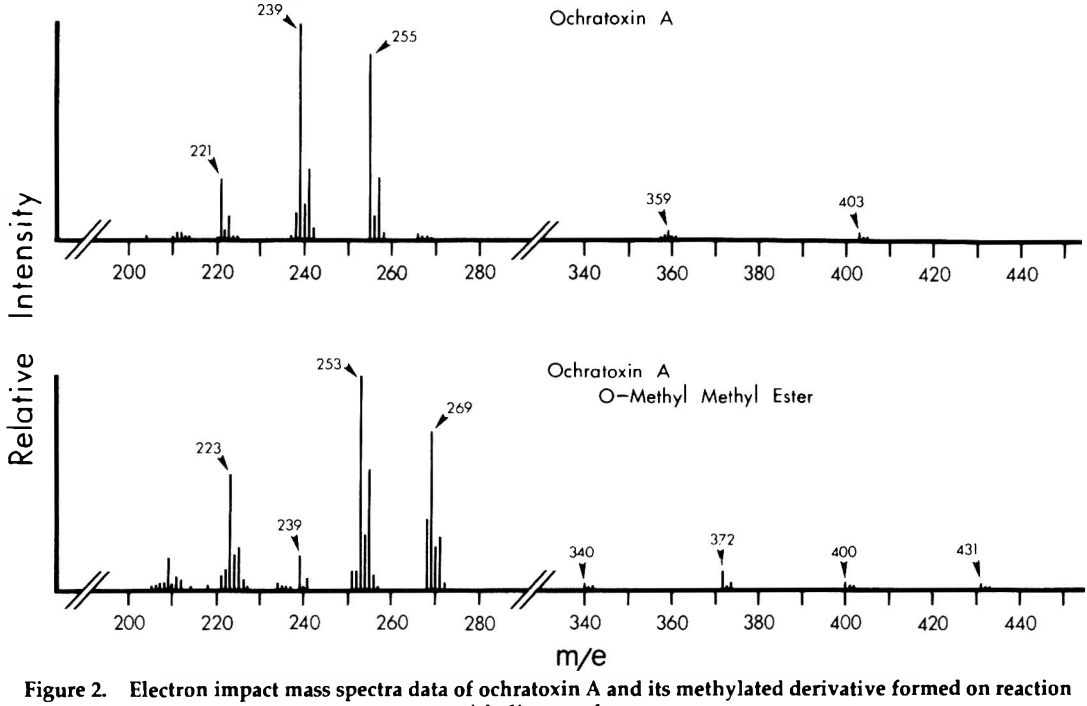

Figure 2. Electron impact mass spectra data of ochratoxin A and its methylated derivative formed on reaction with diazomethane.

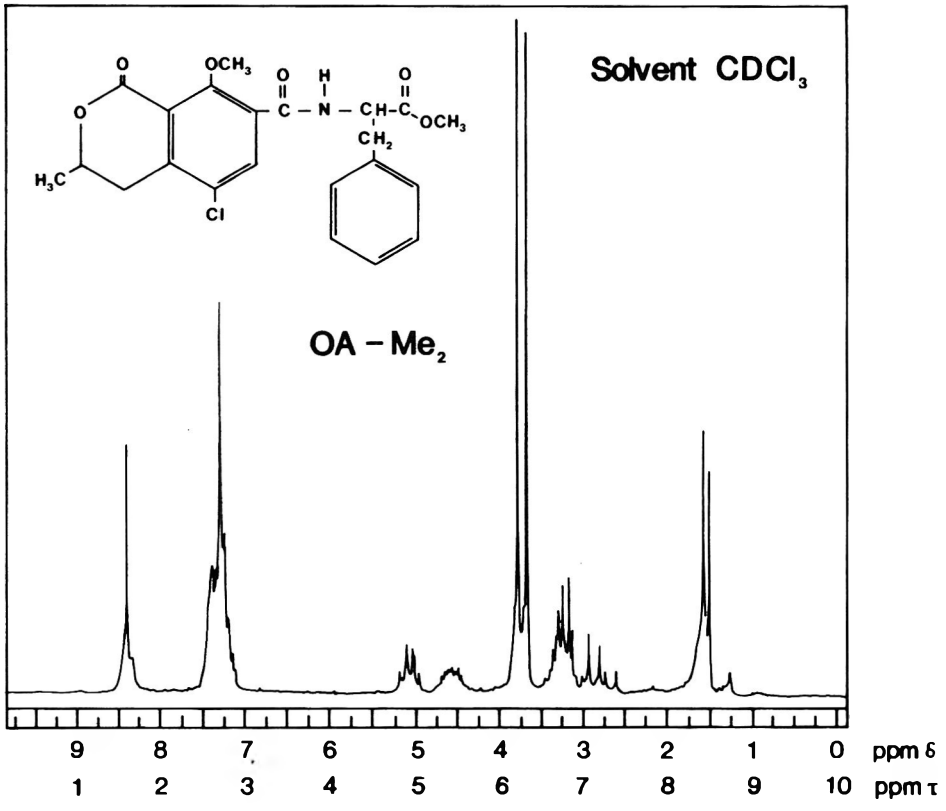

Figure 3. NMR spectrum of the methylated derivative of ochratoxin A formed on reaction with diazomethane. Spectrum recorded in deuterochloroform.

(*OA-Me*$_2$).—Add excess diazomethane to tubes containing OA in 0.1 mL acetone, mix, and let stand 10–20 min. Remove solvent and store dried product in dark at −30°C. Prepare reference standards from dried product in 0.5 mg/mL acetonitrile before use. Make dilutions in acetonitrile as required. Avoid long-term storage.

Extraction and Derivatization of OA from Kidney Homogenates and Plasma

Homogenize chicken kidney samples (1 g) in 4 mL 0.1M phosphoric acid with a Polytron (Brinkmann Instruments). Add 4 mL 0.1M phosphoric acid to 1 mL samples of human plasma. Add OA from standard dilutions in acetonitrile at concentrations ranging from 0.25 to 2.0 μg/g to samples with mixing. Add 4 mL hexane to sample, mix vigorously, centrifuge at 9800 g for 10 min, and discard hexane layer. Repeat hexane procedure, then extract aqueous phase with chloroform (10 mL). Centrifuge at 9800 g for 10 min, repeat chloroform procedure, and discard aqueous layer. Wash chloroform phase with 10 mL water, discard water, then evaporate chloroform to dryness under stream of nitrogen. Dissolve sample residue in acetone (0.1 mL) with mixing. Add excess diazomethane (1–2 mL) to acetone solution, mix, and let stand 10–20 min. Evaporate to dryness under nitrogen and redissolve derivative in 200 μL acetonitrile. Analyze 10 μL aliquots by HPLC. Protect from light, and refrigerate if stored.

Results

Van Der Merwe and colleagues (35), in explaining the structure of ochratoxin A, reacted the mycotoxin with excess diazomethane and reported the formation of an *O*-methyl,methyl ester derivative.

Our studies indicated that diazomethane reacts rapidly with OA. Conversion to OA-Me$_2$ was essentially quantitative after a brief reaction time, although samples were routinely allowed to react for 10–20 min. TLC resulted in an R_f value for OA of 0.089, whereas the R_f was shifted to 0.53

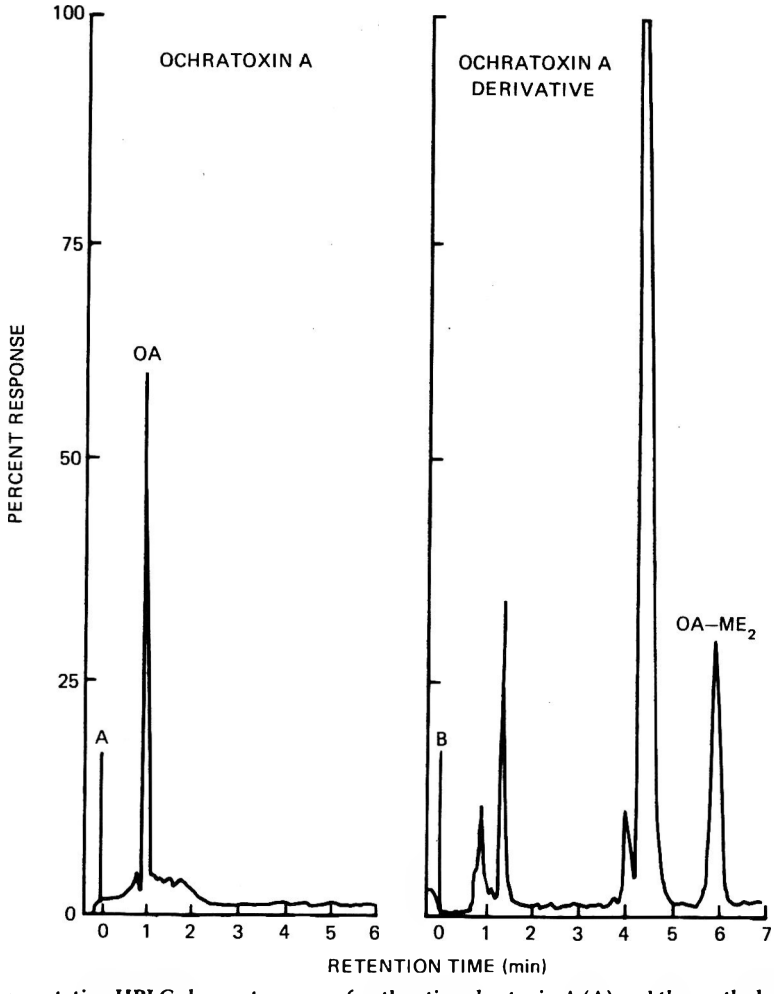

Figure 4. Representative HPLC chromatograms of authentic ochratoxin A (A) and the methylated derivative of ochratoxin A (B); 100 ng (10 μL) injected; see text for conditions.

for OA-Me_2. On the basis of TLC studies in which monomethyl intermediates were not observed in the reaction sequence, it is clear that both the carboxylic acid and the phenolic hydroxyl groups are rapidly methylated by diazomethane, although in the reaction sequence shown in Figure 1, we have perhaps arbitrarily shown the carboxylic acid to be the first site of methylation. The mass spectrum of OA-Me_2 (Figure 2) shows the parent ion at m/z 431, corresponding to the addition of 2 methyl groups to OA. Other ions in the mass spectrum of OA-Me_2 were tentatively assigned as follows: m/z 400 ($m^+ - OCH_3$); 372 ($m^+ - CO_2CH_3$); and 340 ($m^+ - CO_2CH_3$, OCH_3, H; or $m^+ - CH_2C_6H_5$). Major ions at m/z 269 and below result from cleavage of the phenylalanine-isocoumarin linkage (35). The NMR spectra of OA and OA-Me_2 were, as expected, remarkably similar except that in the spectrum of OA-Me_2 (Figure 3), 2 methyl singlets were seen at about δ3.7–3.8. These observations are fully consistent with methylation at the carboxylic and phenolic moieties.

HPLC Analysis of OA-Me_2

Results indicated that both OA and OA-Me_2 could be detected by reverse phase HPLC using a mobile phase of acetonitrile–water (60 + 40) and UV detection at 254 nm. At these conditions, the retention time (R_t) for OA was short (57 s) and detection was hindered by interfering, co-eluting peaks. However, the retention time for the OA-Me_2 product was shifted significantly away from interfering peaks with an R_t of 348 s as shown in Figure 4. The 2 peaks leading OA-Me_2 and eluting with R_t between 4 and 5 min

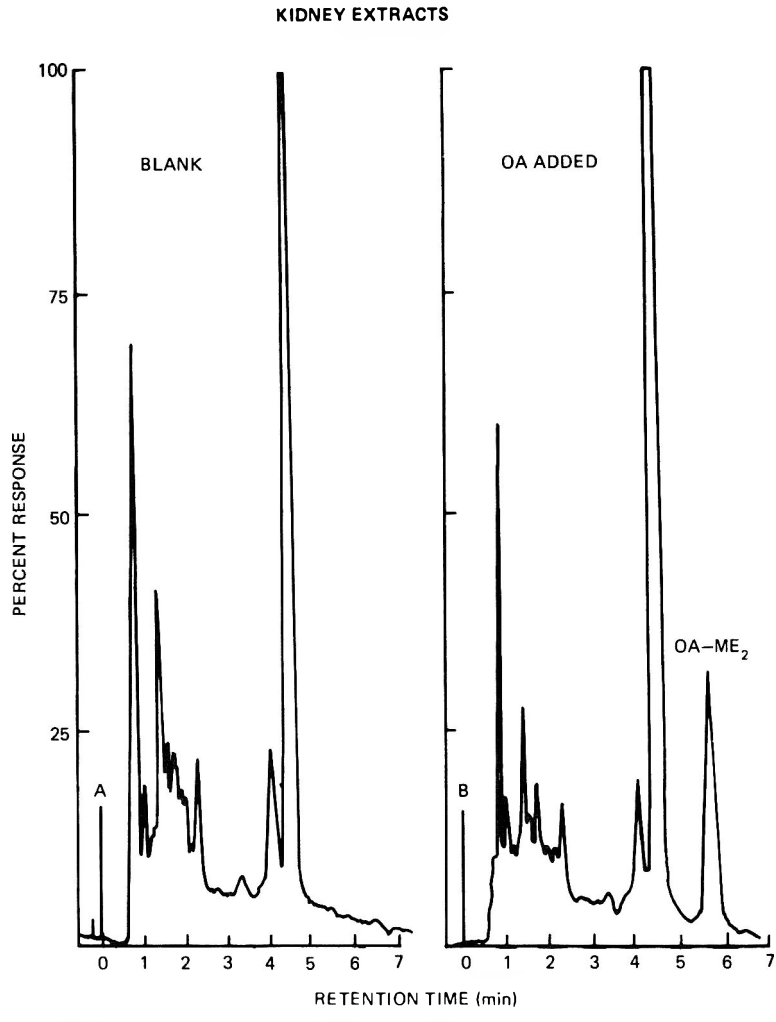

Figure 5. HPLC chromatograms from kidney (A) and ochratoxin-spiked (2.0 μg/g) kidney (B), extracted, derivatized with diazomethane, redissolved in 200 μL acetonitrile, and analyzed by HPLC (10 μL).

were shown to occur as characteristic solvent-diazomethane reaction products, independent of OA. Retention times for OA-Me_2 were very reproducible over a wide range of concentrations. A standard calibration curve for HPLC analysis of OA-Me_2 (which was detectable at levels as low as 3 ng) was linear to 500 ng. The precision of the method was demonstrated by standard deviations of less than ±2% for triplicate assays.

Analysis of OA from Kidney and Plasma as OA-Me_2

Acidified chicken kidney homogenates containing either 0.25, 0.50, 1.0, or 2.0 μg OA/g were extracted with hexane and chloroform and derivatized with diazomethane according to procedures outlined. The recovery of OA as OA-Me_2 from kidney was 71.9–112.3% over a range of concentrations of OA from 0.25 to 2.0 μg/g (Table 1). HPLC response was linear (r = 0.984) for OA-Me_2 from kidney extracts at a range of added OA from 0.25 to 2.0 μg/g kidney. A representative chromatogram of kidney (with and without 2.0 μg OA/g) after extraction and derivatization with diazomethane is presented in Figure 5. The peak with R_t of 348 s corresponded to OA-Me_2.

Human plasma containing added OA was also extracted and derivatized as outlined above. Recovery (Table 1) and separation from inter-

Table 1. Recovery of ochratoxin A from kidney and plasma samples [a]

Ochratoxin A added, μg/g	Mean rec., % [b]	
	Kidney	Plasma
0.25	106.2 ± 6.6	97.2 ± 3.1
0.50	71.9 ± 11.4	108.7 ± 15.5
1.00	112.3 ± 8.2	77.5 ± 5.1
2.00	91.9 ± 5.8	101.1 ± 3.5

[a] Conditions outlined in Methods section.
[b] Data represent average ± standard deviation based on triplicate samples.

fering substances normally present in plasma were good and comparable to that seen with kidney (Figure 6).

Discussion

Results from our studies indicate that HPLC can be used in measuring and confirming OA as the OA-Me_2 derivative. Following simple extraction procedures and derivatization with diazomethane, OA-Me_2 can be accurately quantitated and easily resolved from polar contaminant peaks by reverse phase HPLC because it is significantly less polar than OA and thus has a much longer retention time. This property of OA-Me_2 will be useful in primary analysis or in post-chromatographic confirmation of OA by HPLC either as reported here or in conjunction with other analytical methods of OA analysis.

Since there is strong evidence that OA is an environmentally important nephrotoxin, pos-

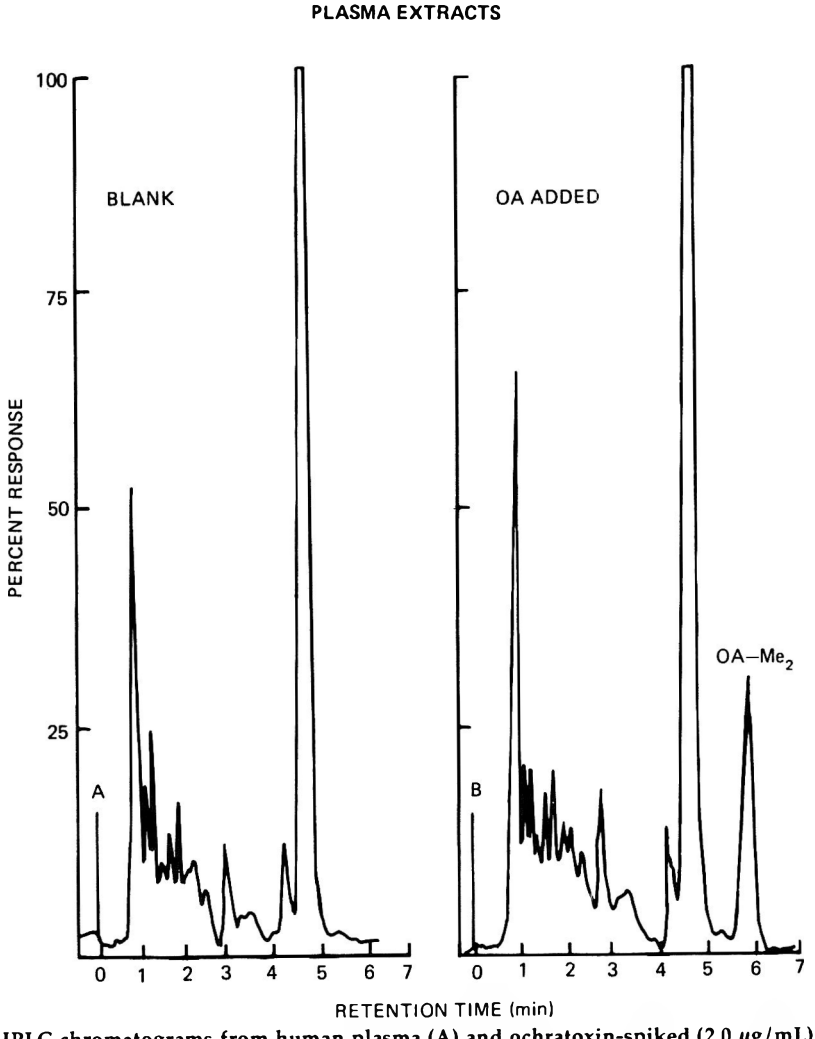

Figure 6. HPLC chromatograms from human plasma (A) and ochratoxin-spiked (2.0 μg/mL) plasma (B), extracted, derivatized with diazomethane, redissolved in 200 μL acetonitrile, and analyzed by HPLC (10 μL).

sibly involved in renal disease of both humans and animals, sensitive analytical methods of quantitation in tissues and biological fluids are highly desirable. Our procedure can be enhanced, after minor modification, to allow tissue detection of OA at much lower levels than presented and is currently being tested for applicability in vivo.

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Simultaneous Detection of Some Fusariotoxins by Gas-Liquid Chromatography

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A method was developed for the simultaneous determination of 6 *Fusarium* mycotoxins (deoxynivalenol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, fusarenon-x, and zearalenone). Cereal samples were first extracted with ethyl acetate, then with a mixture of methanol-water. The crude extracts were combined and purified by silica gel chromatography. The purified extract was reacted with BSTFA (*N,O*-bis(trimethylsilyl)-trifluoroacetamide) to form the derivative, and chromatographed on an SE-52 wall-coated open tubular column. Kováts' retention index was determined for the 6 mycotoxins investigated. Recoveries and standard deviations were determined for pure toxin mixed in cereal. Recovery was 70-80%; relative standard deviation was 10-18%. The method developed was applied to different cereal samples.

In countries of Eastern Europe, including Hungary, *Fusarium* fungus plays an important role in fungal deterioration of feed quality and in animal diseases caused by mycotoxins. Feed may often be infected by several fungus species; moreover, the same fungus species often produces several fusariotoxins. Some analytical methods have been developed for the simultaneous detection of several mycotoxins in a very low concentration range, including simultaneous detection of fusariotoxins.

Stoloff et al. (1) described a method for simultaneous determination of aflatoxin, ochratoxin, zearalenone, sterigmatocystin, and patulin. This method was modified by Scott et al. (2). Roberts and Patterson (3) reported on the simultaneous investigation of 14 toxins. Josefsson and Möller (4) and Pliszczynszka and Juszkiewicz (5) also developed a multimycotoxin screening method. Gimeno (6) described a method for the detection of 10 mycotoxins; Takeda et al. (7) described detection of 14 mycotoxins.

In addition to the thin layer chromatographic testing method, mentioned above, Engstrom and Richard (8) developed a high pressure liquid chromatographic method for multimycotoxin determination.

Takitani et al. (9) described a thin layer chromatographic method for simultaneous *Fusarium* toxin determinations. A suitable color reagent was proposed by authors mentioned above for evaluation of toxins having the trichothecene skeleton. Packed column gas chromatographic methods for simultaneous determination of fusariotoxins were presented by Kamimura et al. (10) and Szathmáry et al. (11). Recently, a glass capillary GLC method was developed for the determination of fusariotoxins in feed (12, 13).

We did not find the TLC determination method (9) and packed column GLC method (10, 11) reliable or sensitive enough. Therefore, we studied the possibility of using capillary GLC for detection of deoxynivalenol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, fusarenon-x, and zearalenone, produced most often by *Fusarium* fungi.

Experimental

Apparatus

(a) *Vacuum rotary apparatus.*—Buchi, Flawil, Switzerland.

(b) *Block thermostat.*—Pierce Chemical Co., Rockford, IL.

(c) *Gas chromatograph.*—Packard Model 427 equipped with FID detector and HP 3390 A integrator. Conditions: injector 240°C, column 180–260°C at 4°/min, detector 260°C, hydrogen carrier gas 40 kPa inlet pressure.

(d) *Screw-cap vials.*—Pierce Chemical Co.

(e) *Capillary glass column.*—Pyrex glass, 8 mm od, 3 mm id, drawn on Hupe Bush capillary drawer (manufactured by Hewlett-Packard) to 12 m × 0.25 mm id. Column was deactivated according to method of Grob et al. (14, 15), and treated by the static method with SE-52 in the phase ratio β = 250 (gas volume/wetting volume).

Reagents and Solvents

(a) *Solvents.*—Commercial preparations (Reanal, Hungary), double-distilled before use.

(b) *Mycotoxin standards.*—1 mg/mL of zearalenone (Supelco 4-6318); 0.5 mg/mL of T-2 toxin (Supelco 4-6322); 0.5 mg/mL of diacetoxyscirpenol (Supelco 4-6315); 0.7 mg/mL of deoxyni-

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valenol, 0.5 mg/mL of HT-2 toxin (Dr. M. Palyusik). All standards prepared in acetone.

(c) *BSTFA.*—*N,O*-Bis(trimethylsilyl)-trifluoroacetamide (Pierce Chemical Co.).

(d) *Silica gel 60.*—Merck, 50–125 μm.

Extraction and Purification

Grind 10 g wheat to grits fineness. Extract ground wheat 2 h at room temperature with 200 mL ethyl acetate, shaking mixture repeatedly during extraction. Filter off organic solution and put aside. Extract residue 2 h at room temperature with 200 mL methanol–water (6 + 4). Combine methanol–water extract with ethyl acetate extract. Dry combined extracts over anhydrous Na_2SO_4. Filter solution from Na_2SO_4 and evaporate on vacuum rotary apparatus to oily liquid residue. Dissolve residue in 2 mL benzene–acetone (2 + 1) and add to 10 × 1 cm column packed with 7 mL silica gel. Wash column with 20 mL benzene to remove lipids. Elute mycotoxins with 20 mL benzene–acetone (1 + 1). Evaporate eluate containing toxins to dryness on vacuum rotary apparatus.

Derivatization

Dissolve residue in 2 mL acetone. Transfer 500 μL acetone solution to screw-cap vial and evaporate in nitrogen atmosphere. Add 100 μL BSTFA reagent to vial, close tightly, and heat 15 min at 60°C. Let mixture cool; then inject 1 μL into gas chromatograph.

Recovery Experiments

Spike 20 g wheat samples at 100 μg fusariotoxins/kg by adding 20 mL of 0.1 μg fusariotoxins/mL working solutions prepared in acetone. Thoroughly mix spiked samples, evaporate solvent under nitrogen stream, and prepare as described under Extraction, Purification, and Derivatization.

Calculations

To calculate results, use the following formula:

$$\text{Toxin, } \mu g/kg = (PA/PA') \times V/(tg_\alpha \times m)$$

where PA and PA' = area of toxin peak and area of internal standard peak, respectively; tg_α = slope of standard curve, concentrations of injected sample vs area of peak; V = volume of silylated sample, μL; m = amount of wheat used in analysis.

Index Calculation

In practice, the index calculation system is widely used. *n*-Alkanes as reference substances form the basis of the system (16). These are chemically inert, and elute from the stationary phases generally used (12).

Table 1. Retention index values of *Fusarium* toxins [a]

Toxin name	Retention index
Zearalenone	2867
T-2 toxin	2822
HT-2 toxin	2809
Diacetoxyscirpenol	2426
Fusarenon-x	2485
Deoxynivalenol	2323

[a] Values were calculated from isothermal temperature run at 200°C.

Mode of calculation:

$$I_x^T = 100\left(n\frac{\log R_x - \log R_z}{\log R_{z+n} - \log R_z} + z\right)$$

where I_x^T = index value of the unknown substance; R_x = adjusted retention time of the unknown substance; R_z = adjusted retention time of the normal alkane with carbon atom number z; R_{z+n} = adjusted retention time of the normal alkane with carbon atom number z + n; z = carbon atom number of reference *n*-alkane, n = difference of the carbon atom number of the 2 *n*-alkanes; T = temperature, °K

For the 6 mycotoxins investigated, Kováts' retention index has been calculated as shown in Table 1.

Results and Discussion

The stationary phase SE-52 was the most suitable of 3 different (SE-30, SE-52, OV-17) materials tested. Separation of DAS, fursarenon-x (FX), and standard T-2 tetraols was not satisfactory on SE-30. However, this phase may be used for a longer time and remains stable. OV-17 stationary phase gives good separation for DAS, FX, T-2 toxin, and HT-2 toxin, but the necessary working temperature (180–280°C) is too high and degrades the stationary phase. The SE-52 has somewhat lower separation efficiency, but it is very stable. After 2 years of use, we obtained good results (see Figure 1).

Naturally occurring fusariotoxins can be detected in combined water–methanol and ethyl-acetate extracts of animal feedstuffs. The capillary technique allows the use of shorter columns, which considerably reduces analysis time; however, if a 15 m long column is used with temperature programming, the analysis time may be as long as 25–30 min, but resolution capacity improves under these conditions (Figure 2).

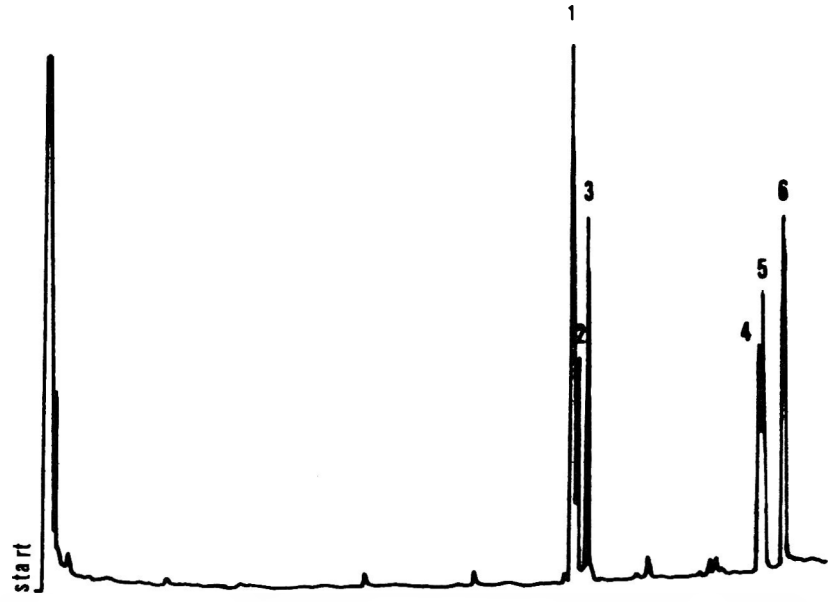

Figure 1. Gas chromatogram of silylated standard mycotoxins: 1, diacetoxyscirpenol (12 ng); 2, fusarenon-x (7 ng); 3, T-2 tetraol (15 ng), I.S.; 4, HT-2 toxin (9 ng); 5, T-2 toxin (10 ng); 6, zearalenone (15 ng).

The sensitivity of the procedure is high enough to detect 100 ppb levels of toxins in feed.

Recoveries and standard deviations calculated from 11 replicates of 100 μg/kg added to the 6 substances investigated in a wheat matrix are shown in Table 2.

The fodder samples studied were selected on the basis of preliminary veterinary evidence of *Fusarium* toxin-positive samples.

In 1980-81, we obtained 23 feed samples. Using the capillary GLC technique, we were able to detect *Fusarium* toxins in 15 samples. Eight samples did not contain *Fusarium* toxins in a detectable quantity. The results are summarized in Table 3. In many cases, several toxins may be

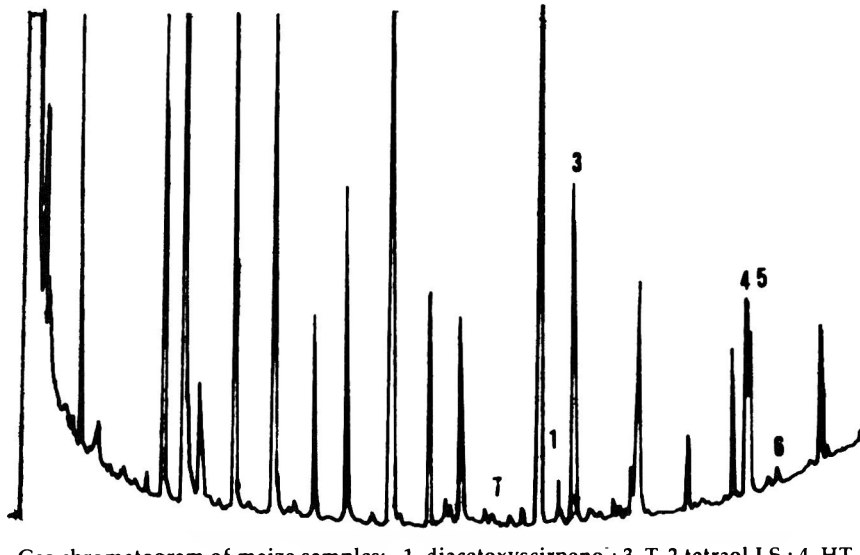

Figure 2. Gas chromatogram of maize samples: 1, diacetoxyscirpenol; 3, T-2 tetraol I.S.; 4, HT-2 toxin; 5, T-2 toxin; 6, zearalenone; 7, deoxynivalenol.

Table 2. Recovery (%) of 6 *Fusarium* toxins from corn

	100 ppb added[a]					
Sample	Zearalenone	T-2 toxin	HT-2 toxin	DAS[b]	DON[c]	Fusarenon-x
1	76	68	70	73	62	67
2	80	74	76	75	71	72
3	78	73	78	81	74	77
4	72	66	71	76	72	74
5	84	79	82	82	73	76
6	86	79	84	89	75	79
7	81	74	80	85	74	72
8	79	70	77	82	70	69
9	76	71	72	76	65	68
10	78	73	74	78	67	69
11	87	81	85	89	73	79
Mean ± SD	79.7 ± 4.5	73.5 ± 4.7	77.2 ± 5.2	80.5 ± 5.5	70.5 ± 4.2	72.9 ± 4.4

[a] All blanks gave 0% recovery.
[b] DAS = diacetoxyscirpenol.
[c] DON = deoxynivalenol.

Table 3. Mycotoxin content (mg/kg) of cereal and feed samples studied

Sample	Type	Zearalenone	T-2 toxin	HT-2 toxin	Diacetoxy-scirpenol	Deoxy-nivalenol
1	Maize	5.7	0.3	0	0.8	0
2	Maize	3.2	1.4	0	0.5	0
3	Wheat	0.2	1.9	0.2	0	0
4	Wheat	0	0.2	0	0	0.5
5	Maize	0	0.4	0	0	1.3
6	Maize	7.5	0	0	2.1	0
7	Maize	3.7	0	0	1.5	0
8	Maize	4.8	0	0	2.0	0
9	Swine food	0	4.1	0	0	0
10	Swine food	0	5.8	0	0	0
11	Maize	1.3	4.4	0.7	0	0
12	Maize	0.7	3.8	0.5	0	0.2
13	Maize	0	0.3	0	0	0
14	Maize	0	0.1	0	0	0
15	Maize	1.7	0.7	0	0.5	0

detected in the same sample. These experimental data support the hypothesis that *Fusarium* infection simultaneously forms several mycotoxins, and the symptoms evidenced in animals result from the toxic effect of several substances. It may also be postulated that several *Fusarium* species are present in the feed sample at the same time. Thus it is possible that zearalenone, T-2 toxin, and HT-2 toxin may occur together, not only, as is common, zearalenone and deoxynivalenol or T-2 toxin and HT-2 toxin.

In some cases, the effect of a single toxin predominated, and only the predominant toxin could be detected in the test; quantities of other toxins were below the limit of detection.

Acknowledgment

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Extraction and Thin Layer Chromatography of Aflatoxin B_1 in Mixed Feeds

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A method was developed for the determination of aflatoxin B_1 in commercially prepared feeds. The method incorporates methylene chloride and citric acid solution extraction, cleanup on a small silica gel column, and thin layer chromatography for quantitation. Commercial turkey starter, catfish chow, medicated pig starter, broiler finisher, rabbit chow, horse feed, rat chow, and dog chow were investigated. The feeds were spiked with naturally contaminated corn at 4 different levels of aflatoxin B_1 (16-130 μg/kg). Three assays were run on each of the 32 combinations of feed and levels of aflatoxin. Mean recoveries were 85.9-92.8% at levels of 16.5, 32.9, 65.8, and 131.6 μg/kg. The relative standard deviation per assay was 18.6%. This method is more rapid and less involved than most previously published methods for mixed feeds.

A new method was developed for the determination of aflatoxin B_1 in commercially prepared feeds because of the great diversity that can occur in mixed feeds. Presently available methods may work very well on some feeds and not at all on others. Other drawbacks must also be considered. One method (1) involves the routine use of a 2-dimensional thin layer chromatography (TLC) that is more time consuming than desired. Another (2) uses a rather complicated purification procedure. Recoveries were only 60-65% for a third method (3). The present method, a modification of the animal tissue method (4), is comparatively simple, rapid, and inexpensive, yielding mean recoveries of aflatoxin B_1 from 85.9 to 92.8%. This research included 8 feeds, each feed spiked at 4 different B_1 levels (16.5, 32.9, 65.8, and 131.6 μg/kg). Three assays were run on each of the 32 combinations of feed and level, and the B_1 level is reported.

Feed samples were spiked with a naturally contaminated grain sample because of the adverse effects of the feed ingredients on recoveries of aflatoxin B_1 in a pure solution. This does not set a precedent (2, 3). Statistically, the standard deviation for recovery values in 9 assays of the spiking grain does not differ significantly from that of the spiked feed samples.

METHOD

Reagents and Apparatus

(a) *Solvents.*—ACS grade glacial acetic acid, acetone, acetonitrile, benzene, dichloromethane, ethyl ether (0.01% ethanol and 1 ppm butylated hydroxytoluene), hexane (boiling range 68-69°C), tetrahydrofuran, and toluene.

(b) *Citric acid solution.*—20%. Dissolve 200 g ACS grade citric acid monohydrate in 1 L water.

(c) *Silica gel 60.*—Merck 7734. Activate 1 h in 105°C oven, add 1% water, and equilibrate overnight.

(d) *Sodium sulfate.*—Anhydrous, granular.

(e) *Diatomaceous earth.*—Hyflo Super-Cel.

(f) *Aflatoxin B_1 standard.*—Prepare in acetonitrile–benzene (2 + 98) to contain 0.5 μg/mL for either visual or densitometric analysis. Store standards in sealed glass ampules, in 0°F freezer until needed. After opening, store in 1 dram vials fitted with Teflon-lined screw caps, and keep in freezer when not in use.

(g) *Wrist-action shaker.*—Burrell, or equivalent.

(h) *Chromatographic columns.*—Glass (1.0 cm id × 50 cm) equipped with Luer nylon stopcock (Bio-Rad Econo-columns), or equivalent.

(i) *Filter paper.*—24 cm S & S No. 560, or equivalent, and 12.5 cm Whatman 934-AH glass microfiber filters, or equivalent.

(j) *Thin layer plates.*—Commercial prepoured plates (20 × 20 cm, Macherey and Nagel Sil G-25 HR), or equivalent.

(k) *Scanning spectrofluorodensitometer.*—Schoeffel SD-3000-3, or equivalent.

(l) *Single-pan balance.*—Sartorius Model 2250 or Mettler P 1200 N, or equivalent.

(m) *Mixer.*—Vortex Model K-500-2, or equivalent.

(n) *Concentrator.*—SMI Model No. 6610 (Scientific Manufacturing Industries, Inc., Emeryville, CA 94608), or equivalent.

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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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Samples

Commercial feeds were obtained locally and ground, as needed, in a Waring blender. Each feed was judged aflatoxin-free after triplicate analysis for aflatoxin B_1 by the AOAC official method for corn (5). Thin layer chromatography (TLC) of these sample extracts was quite difficult because of numerous fluorescent interferences. In some cases an ether predevelopment was sufficient for cleanup, and in others 2-dimensional TLC was necessary. All feeds used were free of aflatoxin B_1. A pre-analyzed (5), naturally contaminated corn sample was then added to the clean feed by weight, according to the B_1 level desired, to obtain the 50 g necessary for a single analysis. The presence of aflatoxin B_1 in the corn was confirmed by AOAC method 26.083 (5).

Extraction

Weigh 50 g ground and blended feed into 500 mL glass-stopper Erlenmeyer flask. Add 20 g Hyflo Super-Cel. Add 10 mL citric acid solution and 200 mL dichloromethane. Shake flask as vigorously as possible on wrist-action shaker (setting between 2.5 and 3 for Burrell) for 30 min. Filter mixture through paper (S & S 560) into 300 mL Erlenmeyer flask containing 10 g Na_2SO_4, and collect 90–100 mL. Gently swirl flask, let contents settle, and refilter solution through glass microfiber filter (Whatman 934-AH) into 100 mL graduate. Collect 40 mL for column chromatography, which represents 10 g of original feed sample.

Column Chromatography

Equip column with small plug of glass wool to provide base for silica gel. Pour column half full with dichloromethane and add 2.0 g silica gel. Add 3–4 mL dichloromethane and slurry with stainless steel (or glass) rod. Drain to settle silica and rinse sides of column with dichloromethane. When silica has settled and column still has ca 3 mL dichloromethane above packing, add 2 g Na_2SO_4 to cap column. Rinse sides of column with dichloromethane. Drain to ca 1 cm above top of column packing. Transfer extract to column and drain through column by gravity, stopping before passing below column cap. Rinse column sides with ca 2.0 mL dichloromethane and drain to column cap. Wash column with 25 mL glacial acetic acid–toluene (1 + 9) (use same graduate that was used for second filtration step), 25 mL tetrahydrofuran–hexane (1 + 3), and 25 mL acetonitrile–ether–hexane (1 + 3 + 6). Discard washes. Elute aflatoxin B_1 with 60 mL acetone–dichloromethane (1 + 4) and evaporate to near dryness under vacuum. Quantitatively transfer to 1 dram vial with Teflon-lined screw cap, and evaporate to dryness under nitrogen for TLC. Avoid overheating dry extract.

Thin Layer Chromatography

Add 500 μL (0.5 mL) acetonitrile–benzene (2 + 98) to dry sample residue in vial from column chromatography above, cap vial, and mix vigorously ca 1 min, preferably on Vortex shaker. Two solvent systems may be used for TLC because some samples may be encountered that result in inadequate separations. Routine solvent system is water–acetone–chloroform (1.5 + 10 + 90). Second system merely substitutes dichloromethane for chloroform, which results in a less polar system that, consequently, leaves interferences at a lower R_f range than aflatoxin B_1. Dichloromethane substitution should not be used if aflatoxin G_1 and G_2 are present because this system changes the order of resolution to B_1, G_1, B_2, then G_2. The lower R_f toxins may be masked by interferences. Solvent proportions may be varied to accommodate changes in laboratory conditions (e.g., use less water in high humidity; use less acetone at higher temperatures).

Preliminary.—Score plate with vertical lines 1 cm apart to result in 20 individual channels. Apply 10 μL of sample in each of 2 channels, superimposing 10 μL B_1 standard on one channel to serve as an internal standard. Also spot 10 μL B_1 standard in a blank channel. One plate will accommodate 8 samples and one standard. After developing plate in appropriate solvent system, air-dry ca 1–2 min, and examine plate under longwave UV light (365 nm). Compare sample aliquot to B_1 standard for bluish fluorescent spot at same R_f range. If present, dilute sample as needed to match standard. Proceed to quantitative plate, scored as mentioned above, applying necessary amount of sample to match B_1 standard.

Visual.—Spot aliquots of standard in amounts of 6, 8, 10, and 12 μL. Spot sample aliquots similarily, judging quantities to be spotted from preliminary plate. Develop plate in appropriate solvent system. Examine plate under longwave UV light in suitable viewing cabinet. Compare chromatograms of sample aliquots with those of standards for presence of single bluish fluorescent spot with R_f and fluorescence pattern similar to those in standards. If aflatoxins are

Table 1. Aflatoxin B_1 recoveries[a] from spiked feeds

Feed	Spiking levels, μg/kg			
	16.5	32.9	65.8	131.6
Turkey starter	15.21 (93)	30.29 (92)	61.67 (94)	123.63 (94)
Catfish chow	14.41 (88)	30.7 (93)	52.45 (80)	117.87 (90)
Pig starter	13.32 (81)	27.38 (83)	56.05 (85)	134.8 (102)
Broiler finisher	17.41 (106)	32.69 (99)	70.93 (108)	112.45 (86)
Rabbit chow	14.86 (90)	30.25 (92)	52.57 (80)	118.56 (90)
Horse feed	14.97 (91)	30.15 (92)	58.39 (89)	126.18 (96)
Rat chow	15.25 (93)	26.41 (80)	56.65 (86)	117.82 (90)
Dog chow	13.72 (83)	30.6 (93)	55.22 (84)	128.93 (98)

[a] Average of 3 assays per level per feed; recovery (%) in parentheses. Least significant ratio (0.05 level) of 2 means is 1.32.

judged present in sample, match fluorescence with one of the standard spots, interpolating between standards if necessary. If aflatoxin zone in sample aliquot is more intense than 6 μL standard spot, suitably redilute sample extract, taking into account amount removed in first TLC, and repeat TLC. Calculate as follows:

$$B_1\ (\mu g/kg) = (S \times Y \times V)/(X \times W)$$

where S = μL aflatoxin standard matching unknown; Y = concentration of standard, μg/mL; V = μL of final dilution of sample extract for TLC; X = μL of sample extract matching S; W = weight of product represented by final extract for TLC (10 g).

Densitometric.—Using scored plate, spot 3 aliquots of B_1 standard and 3 aliquots of each sample (trying to match standard). Develop in appropriate solvent system. Air-dry ca 1–2 min, then scan on densitometer with settings of 365 nm for excitation and 445 nm for emission. Calculate peak areas from strip recorder chart or use in-line computer figures. Calculate as follows:

$$B_1\ (\mu g/kg) = (B \times Y \times V)/(Z \times W)$$

where B = average area of aflatoxin peaks per μL in sample aliquots; Y = concentration of aflatoxin standard, μg/mL; V = μL final volume (500 × dilution) of sample extract for TLC; Z = average area of aflatoxin peak per μL in standard aliquots; W = weight of product represented by final extract for TLC (10 g).

Results and Discussion

This study was originally attempted with a pure solution of aflatoxin B_1 for spiking. After several mixed feeds were spiked and analyzed, it was found that recovery levels were not consistent, ranging from 37 to 147%. Often, within the same feed, the range could vary by 50% or more. Feed additives evidently can have nonreproducible effects on pure aflatoxin. It was decided to redesign the experiments and use a naturally contaminated corn sample for spiking the mixed feeds. Recovery ranges from these samples were satisfactory (Table 1).

The analysis of variance for these data is shown in Table 2. Log B_1 was used because a very wide range in spiking levels (8-fold) was examined.

The analysis of Table 2 indicates the following:

(a) There is no significant variation among feeds in mean B_1 (averaged over 4 levels × 3 samples); the feed mean square of 0.00583 equals the variation among assays.

(b) There is a highly significant linear trend (L_L) with spiking level. Deviations from a linear trend are not significant (L_R).

(c) Regardless of feed, the relation between B_1 level and spiking level is linear. There was no evidence that spiking level interacted with feed.

(d) The standard deviation among assays is $\sqrt{0.00551} = 0.07423$ in log units. The relative

Table 2. Analysis of variance of log B_1 for 8 feedstuffs with 4 spiking levels of B_1

Source of variation	df[a]	Mean square
Feed (F)	7	0.00583 ns
Level (L)	(3)	
Linear L_L	1	11.05269**
Remainder L_R	2	0.00690 ns
F × L interaction	(21)	
FL_L	7	0.00390 ns
FL_R	14	0.00288 ns
Among assays	64	0.00551
	95	

[a] df = degrees of freedom.
** Significant at 0.01 level; ns = not significant.

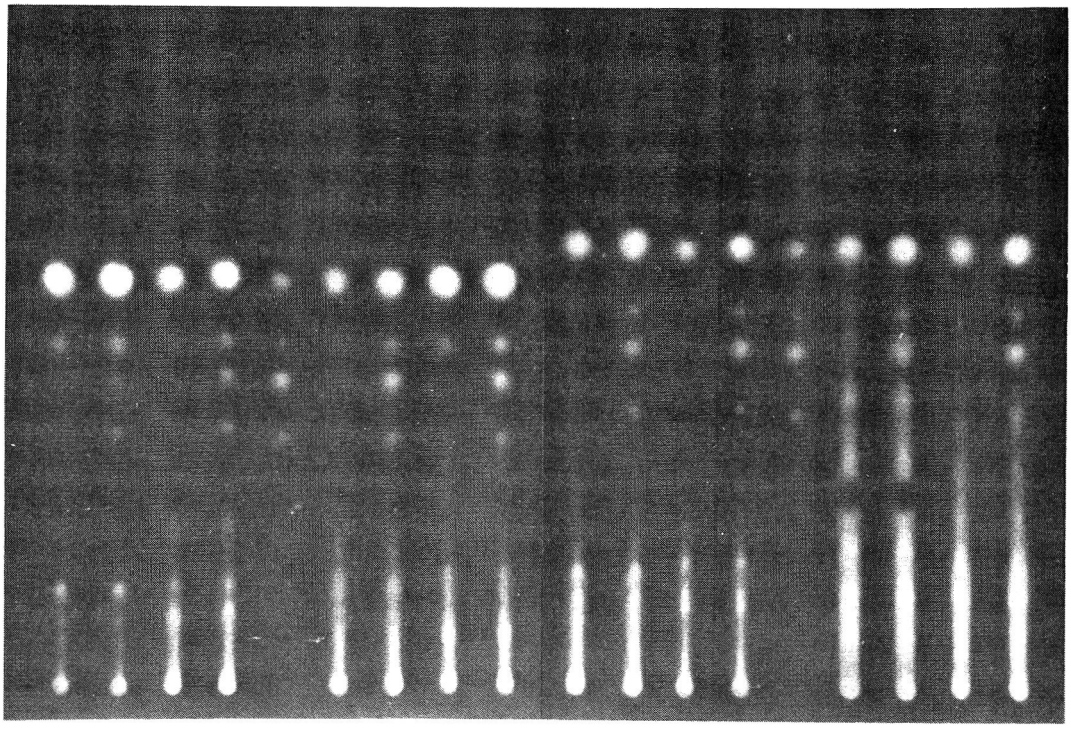

Figure 1. Composite picture of 2 TLC plates showing spiked feed extracts spotted alone and in admixture (even numbered spots) with B_1 standard as follows: 1 & 2 = turkey starter, 131.63 μg B_1/kg; 3 & 4 = broiler finisher, 65.82 μg B_1/kg; 5 & 6 = pig starter, 32.9 μg B_1/kg; 7 & 8 = horse feed, 131.63 μg B_1/kg; 9 & 10 = catfish chow, 32.9 μg B_1/kg; 11 & 12 = dog chow, 32.9 μg B_1/kg; 13 & 14 = rabbit chow, 32.9 μg B_1/kg; 15 & 16 = rat chow, 32.9 μg B_1/kg; S = aflatoxin B_1 standard, 0.5 μg/mL.

standard deviation per assay is $100\ (1-10^{0.07423}) = 18.6\%$.

In addition, there is no significant evidence that variation among assays depended on feed type, although catfish chow and pig starter had somewhat larger variances than other feeds.

The least significant ratio significantly greater than 1.00 at the 0.05 level is 1.322, assuming 3 assays. This value applies to the sample means for a feed-level combination.

The mean B_1 recoveries for all mixed feeds are shown in Table 3. The computed overall relation between spiking level (X) and B_1 was

$$B_1 = 0.86676X^{1.0083}$$

The exponent 1.0083 is not significantly different from unity. If an assumed exponent of unity is used, the model becomes

$$B_1 = 0.8947X$$

Thus, recovery is estimated at 89.5%. In an unpublished study, our mixed feeds method and Romer's mixed feeds method (2) were used to analyze 108 chicken ration samples, 27 of which were positive. The arithmetic coefficient of variation for all positive samples analyzed by the Romer method was 33.3% and by our method 20.9%. The limit of detection of aflatoxin B_1 is less than 2 μg/kg in most feeds with the NRRC method. This was accomplished by spiking blank feed extracts with standard solution and running on a TLC plate.

Figure 1 pictures the various feed extracts on

Table 3. Mean B_1 recovered at 4 spiking levels

Spiking levels, μg/kg	Geometric mean B_1 recovered, μg/kg	Rec., %
16.45	14.78	89.8
32.9	29.44	89.5
65.8	56.54	85.9
131.6	122.18	92.8
Maximum level significantly different from 100		93.3

a TLC plate. Detection of aflatoxins B_1 and B_2 is no problem, but occasionally pig starter has a blue fluorescent zone very near B_1. This can be eliminated by replacing chloroform with dichloromethane in the TLC developing solvent. Then the interfering substance moves ahead of B_1. Determination of G_1 and G_2 would be very difficult due to low R_f materials.

The use of glass fiber filters for the last filtration step is very important. This removes column-clogging fines and permits a fast flow rate that lessens evaporation problems.

Preliminary experimentation with high pressure liquid chromatography (HPLC) was tried on some of the sample extracts, but the results were not satisfactory. We employed a water adduct derivatization with trifluoroacetic acid before injection, and much of the lower R_f material (by TLC) caused interferences by HPLC. This area warrants more research.

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High Pressure Liquid Chromatographic Determination of Xanthomegnin in Grains and Animal Feeds

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A high pressure liquid chromatographic (HPLC) method is described for the determination of xanthomegnin in grains and mixed animal feeds at levels ranging from 150 to 1200 ng/g. This is equivalent to actual amounts of xanthomegnin injected on the HPLC system at from 15 to 120 ng/injection. Xanthomegnin is extracted with chloroform and 0.1M phosphoric acid. An aliquot of the crude extract is purified by column chromatography using a commercially available silica gel cartridge. Xanthomegnin is then separated from the remaining interferences by HPLC with a reverse phase C-8 column, and subsequently determined by absorbance detection at 405 nm. Elapsed time for the method from initial extraction to final HPLC determination is approximately 1 h. Recoveries of xanthomegnin added to grains and animal feeds at levels from 150 to 1200 ng/g averaged 82% with a coefficient of variation of 10.2%.

Xanthomegnin was first reported as a metabolite of the mold *Trichophyton megnini* (1, 2). Xanthomegnin has also been isolated as a metabolite from *T. rubrum* (3, 4), *T. violaceum* (5), *Aspergillus sulphureus* and *A. melleus* (6), *A. ochraceus* (7), and *Pencillium viridicatum* (8). Several of these xanthomegnin-producing molds have been found as part of the natural mycoflora of grains used as foods and feeds (9) as well as of hams and sausages (10). The presence of this metabolite in moldy contaminated foods and feeds may, therefore, be suspected.

Xanthomegnin has been implicated as a causative agent responsible for liver and renal damage in swine (11) and has been shown to be toxic when fed to mice and rats (12). Several diseases have been suspected to be linked with this compound (P. Krogh, Dept. of Microbiology, Royal Dental College, Copenhagen, Denmark, private communication, 1981). Hence, good, reliable methods for sensitive detection of xanthomegnin are needed.

Only one method has been reported for the determination of xanthomegnin (13). This method, applied to the analysis of corn samples, has a limit of detection of 0.75 mg/kg and an overall recovery of added xanthomegnin of less than 50%.

We were planning to analyze several hundred samples of grains for xanthomegnin as part of a survey of the presence of mycotoxins in farm grains, so the development of a method with a limit of detection of at least 0.20 mg/kg and an average recovery of not less than 75% was considered essential to obtain a true evaluation of the extent of contamination.

METHOD

Apparatus

(a) *Reservoir.*—10 mL glass syringe with a Luer tip or polypropylene Econo-Column (Bio-Rad Laboratories, Richmond, CA 94804) to serve as Sep-Pak column reservoir.

(b) *Silica gel cartridge.*—Sep-Pak (Waters Associates, Inc., Milford, MA 01757).

(c) *Pressure applicator.*—Squeeze bulb with check valve in one end and rubber tubing attached to other end (atomizer bulb set No. 56325-000, VWR Scientific, Inc., Chicago, IL 60104), connected to short piece of glass tubing. To other end of glass tubing a 1-hole rubber stopper is attached to fit the syringe barrel or Econo-Column. This assembly acts as source of positive pressure to force eluants through the cartridge.

(d) *Liquid chromatographic equipment.*—Beckman model No. 100A pump with high pressure loop injection valve fitted with 20 μL sample injection loop, Beckman 5 μm octyl (C-8) column, 4.6 mm × 25 cm, and Beckman model No. 100-40 variable wavelength UV-visible absorbance detector set at 405 nm (Beckman Instruments, Inc., Berkeley, CA 94710).

(e) *Recorder.*—Kipp & Zonen model No. BD41 strip chart recorder with 10 mV input (Beckman Instruments, Inc.).

(f) *Metal Swinney filter holder.*—13 mm (Millipore Corp., Bedford, MA 01730, No. 1xx30 012 00). Holder fitted with glass microfiber filter paper, 13 mm, Gelman type A/E, or equivalent

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(Gelman Instrument Co., Ann Arbor, MI 48106).

(g) *Wrist-action shaker.*—Burrell model No. 75 (Burrell Corp., Pittsburgh, PA 15219).

(h) *TLC developing tank.*—Metal TLC chamber with solvent trough and tight fitting lid.

(i) *Precoated silica gel plates.*—0.25 cm layer silica gel GHR (Brinkmann Instruments, Inc., Westbury, NY 11590).

Reagents

(a) *Solvents.*—Chloroform, distilled in glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). ACS reagent grade acetonitrile, benzene, anhydrous ethyl ether, glacial acetic acid, hexane, methanol, phosphoric acid, ammonium hydroxide, HPLC grade water.

(b) *Mobile phase.*—Dissolve 1.4 g KH_2PO_4 in 450 mL HPLC grade water. Add 550 mL acetonitrile. Adjust pH to 3 with phosphoric acid.

(c) *Xanthomegnin.*—Obtained from Michael Stack, Bureau of Foods, Food and Drug Administration, Washington, DC 20204.

Stock solution.—Dissolve crystalline xanthomegnin in chloroform–acetic acid (99 + 1) to obtain concentration of 30 μg/mL. When stored in amber glass in a freezer, this solution is stable ca 2 months. *HPLC working standard solutions.*—Transfer appropriate amounts of stock solution into amber glass vials to give following amounts of xanthomegnin per vial: 1.5, 3, 6, and 12 μg. Evaporate working standards to dryness under stream of nitrogen at ≤40°C in dim light. These films are stable indefinitely when kept in a freezer. Immediately before use, dissolve contents of each vial with 2.0 mL mobile phase. These solutions are stable ca 1 working day.

Sample Preparation and Extraction

Note: Because of the labile nature of xanthomegnin, it is desirable to carry the analysis through from start to finish without stopping. If extracts or cleaned-up solutions must be stored for a short time, it is necessary to protect them from undue exposure to light. Keep the extracts in the dark or store in amber glass.

Grind representative sample to pass 2 mm (U.S. No. 10) sieve. Weigh 25 g sample into 250 mL glass-stopper Erlenmeyer flask. Add 10 mL 0.1M phosphoric acid and 100 mL chloroform. Stopper and shake 30 min, using wrist-action shaker set at moderate rate. Filter sample through fluted paper (S&S 588 or equivalent) into 100 mL Erlenmeyer flask.

Column Chromatography

Transfer 20 mL aliquot of filtrate to 50 mL beaker and evaporate to 2–5 mL at ≤40°C under stream of nitrogen. Attach column reservoir to longer end fitting of silica gel Sep-Pak cartridge. Quantitatively transfer condensed aliquot to column reservoir with a total volume of ≤5 mL chloroform. Use pressure applicator to pump filtrate through column at flow rate of 1–2 drops/s (do not permit column to go dry) and discard eluate. Wash column with 10 mL hexane–ether (3 + 1) at same flow rate as described above, and discard eluate. Elute xanthomegnin from column with 10 mL chloroform–acetic acid (98 + 2) into 14 mL amber glass vial at same flow rate as above. Evaporate final eluate just to dryness (no odor of acetic acid) at ≤40°C under stream of nitrogen. Dissolve extract in 1.0 mL chloroform. Transfer 500 μL to second amber glass vial for HPLC determination. Retain extract in first vial for confirmation. Evaporate contents of second vial just to dryness at ≤40°C under stream of nitrogen. This dry extract may be stored, if necessary, for several days in refrigerator without any loss of xanthomegnin. Just before HPLC determination, dissolve contents of this vial in 500 μL HPLC mobile phase. Clarify solution if necessary by filtering through Swinney filter holder fitted with glass microfiber paper.

High Pressure Liquid Chromatography

Set HPLC flow rate to 1.0 mL/min and UV-visible absorbance detector to 405 nm. Let pump and detector run ca 30 min before use to permit equilibration of HPLC column and stabilization of detector.

Inject full loop (20 μL) of each standard and construct standard curve from plot of peak height vs concentration to verify performance of system. This should be done daily. Retention time of xanthomegnin is 7–8 min.

Inject full loop (20 μL) of sample solution. Determine concentration to xanthomegnin in sample extract directly from standard curve. Calculate amount of xanthomegnin in sample by the following equation:

$$\text{Xanthomegnin, ng/g sample} = (A \times B/C) \times 1000$$

where A = concentration, μg/mL, of xanthomegnin in sample extract; B = volume, mL, of sample extract; C = amount of sample represented by extract (5 g).

TLC Confirmation of Identity

This confirmation of identity is essentially the same as that described by Stack et al. for the HPLC determination of xanthomegnin in corn (13).

Evaporate contents of first vial just to dryness at ≤40°C under stream of nitrogen. Using calculated result for amount of xanthomegnin found in sample as guide, dissolve contents of vial in chloroform such that concentration of xanthomegnin is ca 5 ng/μL. (Example: Vial contains 2.5 g sample. Calculated result = 150 ng xanthomegnin/g sample, and vial therefore contains 375 ng xanthomegnin; 375 ng xanthomegnin ÷ 5 ng xanthomegnin/μL = 75 μL chloroform.) Spot 25 μL of this solution 4 cm from bottom of TLC plate. Spot 5 μL xanthomegnin stock solution 1 cm away on each side of sample spot. Develop TLC plate with benzene–acetic acid–methanol (90 + 5 + 5) in an unlined and unequilibrated tank until solvent front reaches height of 11–12 cm above spotting line. Let plate air-dry in fume hood for ca 5 min. Expose plate to ammonia fumes for 5 min. Red-to-violet spot in sample at same R_f as standard spot is positive confirmation of presence of xanthomegnin in sample.

Results and Discussion

As part of this research, xanthomegnin standard stock solution was added at levels from 150 to 1200 ng/g to samples of corn, oats, and mixed feed after grinding and before extraction. Recoveries of xanthomegnin from these samples range from 70 to 100% with an average of 82% and a coefficient of variation of 10.2% (Table 1). Chromatograms resulting from an injection of 30 ng xanthomegnin standard and an injection of a mixed animal feed sample spiked at the 300 ng/g level and taken through the method are shown in Figure 1. A blank sample chromatogram is also shown.

The extraction and cleanup used in this method are based on the method described by Stack et al. (13). As stated there, the instability of xanthomegnin in solution resulted in low and variable recoveries. To test this premise, xanthomegnin was dissolved in methanol, acetonitrile, dichloromethane, and chloroform in amber glass vials to give a concentration of 6 μg/mL in each vial. These solutions were allowed to stand in the dark for 3 days. The solvents were then evaporated at 40°C under a stream of nitrogen. The resulting dry films were dissolved in 1.0 mL HPLC mobile phase and injected onto the HPLC system. The peak heights were compared to an equivalent standard which was freshly prepared as described in the method. None of the xanthomegnin residues from the test solvents gave significantly different results for measured peak heights from that of the freshly prepared standard. Therefore, we conclude that xanthomeg-

Table 1. Recovery of xanthomegnin from spiked commodities

Spike level, ng/g	Rec., %
Corn	
150	77
	83
	79
300	85
	87
	84
600	86
	86
	85
1200	88
	82
	87
Mean 84	
CV, % 4.0	
Oats	
150	78
	77
	87
300	100
	80
	77
600	81
	72
	70
1200	79
	74
	70
Mean 79	
CV, % 10.5	
Animal Feed	
150	77
	70
	70
300	83
	100
	100
600	72
	82
	78
1200	91
	92
	93
Mean 84	
CV, % 13.0	
Overall mean = 82	
Overal CV, % = 10.2	

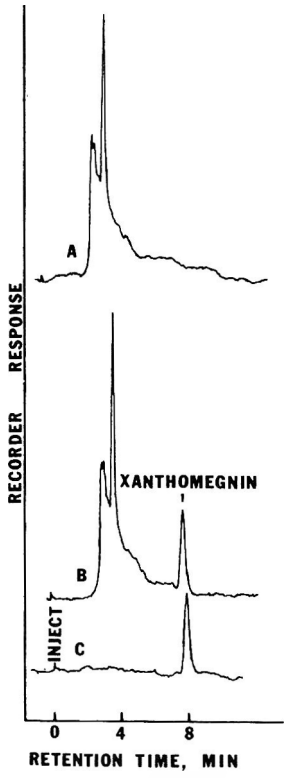

Figure 1. Chromatograms of sample and standard injections of xanthomegnin. (A) Blank sample, mixed animal feed; (B) spiked sample, 300 ng/g level, mixed animal feed; (C) standard xanthomegnin, 30 ng injected. HPLC conditions as given in method.

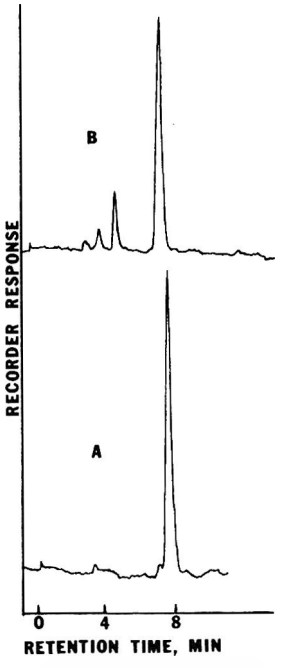

Figure 2. Chromatograms of standard xanthomegnin, 120 ng injected. (A) Xanthomegnin stored at acidic pH (pH 3.0); (B) xanthomegnin stored at basic pH (pH 9.0). HPLC conditions as given in method.

nin dissolved in solvents and stored in the dark is stable for several days. However, it was noted that xanthomegnin dissolved in the HPLC mobile phase began to decompose by the next day. This is probably due to the presence of water in the mobile phase.

To test the effect of pH on the stability of xanthomegnin in solution, 6 μg xanthomegnin was placed in each of 2 amber vials and dissolved in methanol. The solution in one vial was made basic to pH 9 with ammonium hydroxide and that in the other vial was acidified with acetic acid to pH 3. The solutions were allowed to stand for 30 min and then all solvent was evaporated at 40°C under a stream of nitrogen. The resulting dry films were dissolved in the HPLC mobile phase and injected onto the HPLC system. The chromatogram of xanthomegnin stored in acidic solution gave one peak of quantitative yield corresponding to a standard of the same concentration. The chromatogram of xanthomegnin stored in basic solution gave 4 smaller peaks, indicating decomposition (Figure 2).

It was noted that xanthomegnin undergoes a color change from yellow in acidic or neutral solution to red in basic solution. This color change is reversible. Xanthomegnin also undergoes a color change from yellow in neutral solution to red when placed on silica gel. It is therefore suggested that the silica gel used for column chromatographic cleanup of sample extracts for determination of xanthomegnin is at least in part responsible for the loss of xanthomegnin. The longer the residence time of xanthomegnin on a silica gel column, the greater the loss appears to be. During the development of this method, several variations of silica gel column chromatography for sample cleanup were evaluated, and the Sep-Pak procedure described consistently gave the best recoveries. This is most likely due to the small, compact column, which contains only about 2 g silica gel, and to the short residence time of xanthomegnin on the column. The elapsed time from sample application to elution of xanthomegnin is about 10 min.

It is known that some quinones are sensitive to exposure to light. To test if exposure to light

has an adverse effect on the stability of xanthomegnin, a dry film of xanthomegnin in clear glass vials was exposed to normal fluorescent laboratory lighting and also stored in amber glass vials in the dark for 24 h. The lights in the laboratory were turned off overnight. Actual exposure to bright light was about 12 h. The films in the vials were then dissolved in the HPLC mobile phase and injected onto the HPLC system. The xanthomegnin kept in the light showed an average loss of 30% when compared with that kept in the dark.

The method reported here takes into consideration the results of our studies on the effects of solvents, pH, and light on the stability of xanthomegnin. The sample analysis takes an average of 1 h to complete and is designed to reduce to a minimum the exposure of the sample extract to light and a pH above 7. This method has been extensively used in our laboratory for over a year and has given consistent and reproducible results.

Acknowledgments

We thank Michael Stack, Food and Drug Administration, Bureau of Foods, Washington, DC, for providing the standard xanthomegnin used in this work and Leo J. Lipinski, Jr and Eddie J. Rigsby, Food and Drug Administration, New Orleans District Laboratory, for performing some of the recovery studies.

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PLANTS

Derivation of Fluorometric Chlorophyll and Pheophytin Equations

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Equations for calculation of chlorophyll a and pheophytin a by fluorometry have been cited in a number of publications. None of them, however, includes the derivation of the equations to substantiate their validity. The derivation is given here to let the user apply the equations with confidence.

Scientific literature contains quite a number of publications dealing with the determination of chlorophyll *a* in environmental samples by using fluorometry and improvements of the standard method. None of the publications, however, includes the derivation of the 2 equations used in the calculation process: one for the concentration of chlorophyll *a* and the other for the concentration of pheophytin *a* (1–3). *Standard Methods for the Examination of Water and Wastewater* (3) gives these equations as

$$\text{Chlorophyll } a, \text{ mg/cu. m} = Fs \frac{r}{r-1}(Rb - Ra)$$

$$\text{Pheophytin } a, \text{ mg/cu. m} = Fs \frac{r}{r-1}(rRa - Rb)$$

where Fs = calibration factor of sensitivity setting S to convert fluorometric readings to concentrations of chlorophyll *a*; Rb = fluorescence of extract before acidification; Ra = fluorescence of extract after acidification; r = ratio of Rb/Ra.

Derivation

The following derivation of the equations (using some different symbols), which substantiates the validity of the published equations, should be of interest to analytical chemists who use the chlorophyll *a* method.

Definition of symbols:

F = fluorescence of sample
R = fluorescence of pure chlorophyll *a* standard
Fb and Rb = total fluorescence before acidification
Fa and Ra = total fluorescence after acidification
Fc and Rc = fluorescence of chlorophyll *a*
Fp and Rp = fluorescence of pheophytin *a*
Fcp and Rcp = fluorescence of chlorophyll *a* converted to pheophytin *a* by acidification
S = calibration factor for appropriate sensitivity setting

A pure chlorophyll *a* standard contains only chlorophyll *a* and no pheophytin *a*, but a chlorophyll *a* sample may contain pheophytin *a*. The ratio of an unacidified pure chlorophyll *a* standard to an acidified pure chlorophyll *a* standard in fluorescence can be considered constant for each analytical setup (1, 3, 4) and is represented as:

$$\frac{Rb}{Ra} = r$$

The fluorescence of a sample before acidification is described by:

$$Fb = Fc + Fp \quad (1)$$

Solving for Fc,

$$Fc = Fb - Fp \quad (2)$$

The fluorescence of a sample after acidification is described by:

$$Fa = Fp + Fcp \quad (3)$$

Solving for Fcp,

$$Fcp = Fa - Fp \quad (4)$$

If $\frac{Rb}{Ra} = r$, then $\frac{Fc}{Fcp} = r$, and

$$\frac{Rb}{Ra} = \frac{Fc}{Fcp} = \frac{Fb - Fp}{Fa - Fp} \quad (5)$$

Solving Equation 5 for Fp yields

$$Fp = \frac{RaFb - RbFa}{Ra - Rb} \quad (6)$$

which is the fluorescence of pheophytin *a* in the sample. The concentration of pheophytin *a* in the sample is obtained by using a pure chlorophyll *a* standard. When this standard is acidified, all chlorophyll *a* is converted to pheophytin *a*, whose fluorescence is only 42% that of chlo-

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rophyll *a* (1). When, therefore, the concentration of pheophytin *a* is calculated using a pure chlorophyll *a* standard, the fluorescence of pheophytin *a* must be multiplied by *Rb/Ra*. Equation 6 then becomes:

$$Fp = \frac{Rb}{Ra}\left(\frac{RaFb - RbFa}{Ra - Rb}\right), \quad \frac{Fb}{Fa} \leq \frac{Rb}{Ra} \qquad (7)$$

Multiply numerator and denominator by −1:

$$Fp = \frac{Rb}{Ra}\left(\frac{RbFa - RaFb}{Rb - Ra}\right)$$

$$= \frac{Rb}{Rb - Ra}\left(\frac{RbFa - RaFb}{Ra}\right)$$

Divide numerator and denominator by *Ra:*

$$Fp = \frac{\frac{Rb}{Ra}}{\frac{Rb - Ra}{Ra}}\left(\frac{Rb}{Ra}Fa - Fb\right)$$

$$= \frac{\frac{Rb}{Ra}}{\frac{Rb}{Ra} - 1}\left(\frac{Rb}{Ra}Fa - Fb\right)$$

Substitute *r* for *Rb/Ra*

$$Fp = \frac{r}{r - 1}(rFa - Fb)$$

The concentration of pheophytin *a* is then described by:

$$\text{Pheophytin } a = \frac{r}{r - 1}(rFa - Fb)S \qquad (8)$$

Substituting Equation 6 into Equation 2 yields

$$Fc = Fb - \left(\frac{RaFb - RbFa}{Ra - Rb}\right) \qquad (9)$$

$$= \frac{RaFb - RbFb - RaFb + RbFa}{Ra - Rb}$$

$$= \frac{-RbFb + RbFa}{Ra - Rb}$$

Multiply numerator and denominator by −1:

$$Fc = \frac{RbFb - RbFa}{Rb - Ra}$$

$$= \frac{Rb}{Rb - Ra}(Fb - Fa)$$

Divide numerator and denominator by *Ra:*

$$Fc = \frac{\frac{Rb}{Ra}}{\frac{Rb}{Ra} - 1}(Fb - Fa)$$

Substitute *r* for *Rb/Ra:*

$$= \frac{r}{r - 1}(Fb - Fa)$$

The concentration of chlorophyll *a* is then described by:

$$\text{Chlorophyll } a = \frac{r}{r - 1}(Fb - Fa)S \qquad (10)$$

The above derivation arrived at the same equations that are being recommended in a number of publications. Analytical chemists can, therefore, use the equations with confidence.

The above derivation shows the validity of the published equations only. Interferences by other chlorophylls and derivatives should be considered separately.

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DRUGS IN FEEDS

Effect of Citric Acid on Inhibition of Chlortetracycline Activity by Magnesium Ions

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The antimicrobial activity of chlortetracycline (CTC) can be inhibited by magnesium ions; thus the result of CTC turbidimetric assay may be lower than guarantee if large amounts of magnesium ions are present in the feed extract. Under controlled conditions, citric acid can be used to chelate the excess magnesium ions without affecting cell growth or CTC activity, thereby eliminating the magnesium interference.

The official methods for determination of chlortetracycline (CTC) in feeds are the plate agar diffusion method (1) and the turbidimetric method (2). Since the introduction of the Autoturb® system (3), the latter procedure has been greatly improved in both speed and accuracy. However, even with the aid of the Autoturb system, the turbidimetric method is not without its limitations.

A major limitation of the turbidimetric method is its susceptibility to interference by other components in the feed samples. Interference can be caused by compounds that change the growth rate of the test organism and thus affect the assay values.

The turbidimetric assay values of CTC may be biased low if a feed sample contains a high level of magnesium. Magnesium ions can affect the turbidimetric assay in 2 ways: (i) It promotes the growth of the test organism; (ii) it inactivates CTC activity by interfering with the binding between CTC and ribosome (4). It was shown previously (4) that the growth promotion effect can be corrected if the assay broth is supplemented with extra 1mM magnesium ions. Several magnesium chelating agents were tested for their ability to circumvent the second effect of magnesium ions on the CTC turbidimetric assay. In the present report, we show that, under controlled conditions, citric acid can completely eliminate the interference of CTC turbidimetric assay by magnesium ions. Furthermore, at high concentrations, citric acid inhibits growth of the test organism and also enhances CTC activity.

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METHOD

Apparatus and Reagents

(a) *Automatic turbidimetric system.*—Autoturb® (Elanco Products Co.)

(b) *CTC standard solutions.—Stock solution.*—250 μg/mL. Accurately weigh 0.250 g CTC·HCl USP Reference Standard. Dissolve in 0.01N HCl and dilute to 1 L with 0.01N HCl. Dilute aliquots of stock solution with pH 4.5 phosphate buffer to obtain concentrations of 4 and 8 μg/mL.

(c) *Dilution buffer.*—pH 4.5 phosphate buffer. Dissolve 13.6 g anhydrous KH_2PO_4 in water and dilute to 1 L.

(d) *Assay broth.*—Difco antibiotic medium 3. Dissolve 29.75 g medium in 1 L water and use immediately.

(e) *$MgSO_4$ solution.*—1M. Dissolve 120 g $MgSO_4$ in dilution buffer and dilute to 1 L.

(f) *Citric acid solution.*—1M. Dissolve 21 g citric acid monohydrate (molecular weight = 210) in dilution buffer and dilute to 100 mL.

(g) *Test organism.—Staphylococcus aureus*, ATCC 9144.

(h) *Preparation of inoculum.*—Inoculate 10 mL assay broth with 1 loop of *S. aureus* from slant culture. Incubate overnight at room temperature. Use 10 mL culture per liter of assay broth. The cell concentration before incubation was 1.0×10^7 cells/mL or had a percent transmission value of 98%.

Preparation of Samples

Mix 1 mL CTC standard solution at either 4 or 8 μg/mL with appropriate amount of 1M $MgSO_4$ and/or 1M citric acid so that, after dilution to 10 mL with phosphate buffer (pH 4.5), the final concentrations in each of these sample tubes are as indicated in the figures. These samples are automatically diluted with inoculated assay broth, incubated to allow cell growth, and analyzed for percent transmission by the Autoturb spectrophotometric reader. Calculate equivalent CTC concentration or percent CTC inhibition in each sample by interpolating from standard CTC

dose-response curve determined in the same experiment.

Results and Discussion

Effect of Citric Acid on Inhibition of CTC Activity by Magnesium Ions

As previously reported from this laboratory (4), up to 50% of the antimicrobial activity of CTC can be inhibited by exposing the drug to magnesium ions. It has been suggested by several investigators (5, 6) that magnesium ions are normally involved in the binding of tetracyclines to ribosomes, resulting in the inhibition of protein synthesis. It is known that tetracyclines can form chelates with magnesium ions (7, 8); therefore, extra magnesium ions may interfere with the binding of tetracyclines to ribosomes. If this is the mechanism by which magnesium ions inhibit CTC activity, one would expect that other cation chelating agents would reduce the CTC inhibition exerted by excess magnesium ions.

Results obtained by using EDTA (ethylenediaminetetraacetic acid) as a chelating agent were inconclusive; it was so effective in chelating magnesium ions that cell growth was inadvertently inhibited (4). Alternatively, citric acid was used at concentration levels where the cell growth was unaffected. When increasing amounts of citric acid were added to magnesium-treated CTC standard solution, the CTC activity was gradually restored (Figure 1, Panel B). At the highest citric acid concentration used (100mM), essentially 100% of the CTC activity was recovered. Because each citric acid molecule carries 3 negative charges at neutral pH and each magnesium ion carries 2 positive charges, one would expect that it would take 2 moles of citric acid to chelate 3 moles of magnesium ions. This corresponds quite well with our observation here; namely, 100mM citric acid completely eliminates the effect of CTC inhibition exerted by 150mM magnesium ions. Figure 1 also shows that when the same amount of citric acid and 150mM $MgSO_4$ were added to the inoculated media without the addition of CTC, the percent CTC activity remains zero, indicating cell growth was unaffected by $MgSO_4$ and citric acid at these concentration levels.

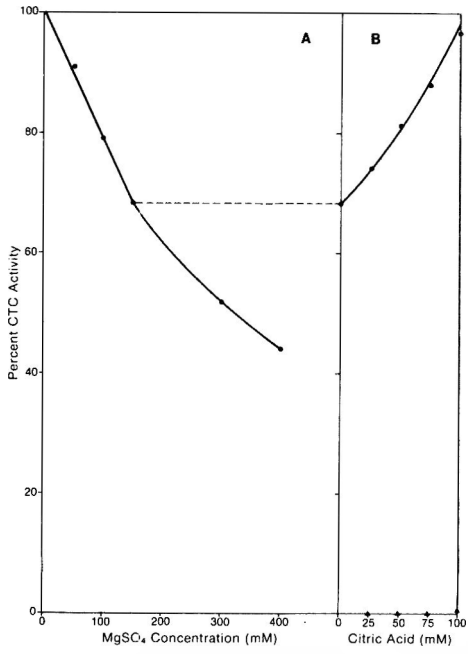

Figure 1. Inhibition of CTC activity by $MgSO_4$ (Panel A), and recovery of this inhibition by citric acid (Panel B). Bottom line (▲-▲-▲) represents samples containing 150mM $MgSO_4$ and increasing amounts of citric acid without CTC.

Enhancement of CTC Activity by Citric Acid

In the course of investigating the effect of citric acid on CTC inhibition by magnesium ions, we found that if citric acid alone was added to standard CTC solution, the extent of growth inhibition was more than if citric acid was not added. This is not surprising; citric acid alone, at higher concentration, can inhibit growth, probably by chelating some of the magnesium ions. However, the amount of growth inhibition resulting from CTC plus citric acid is higher than the sum of individual contribution from each of these 2 components. This is shown in Figure 2 in which growth inhibition was expressed as equivalent CTC concentrations. The bottom line shows that when citric acid concentration exceeds 100mM, growth inhibition can be observed without the addition of CTC. The upper line shows the result if standard CTC solution (at 0.4 μg/mL) was also added. This line shows a steeper overall slope than the line for citric acid alone. The dotted line is the normalized curve obtained by subtracting the lower line from the upper line. As evident from the normalized line, the CTC activity is enhanced by the addition of citric acid. At the highest citric acid concentration (300mM), CTC activity can be enhanced over 35% (from 0.4 to 0.55 μg/mL).

The mechanism of the enhancement of CTC activity by citric acid is unclear. One cannot attribute this phenomenon to changes in cell

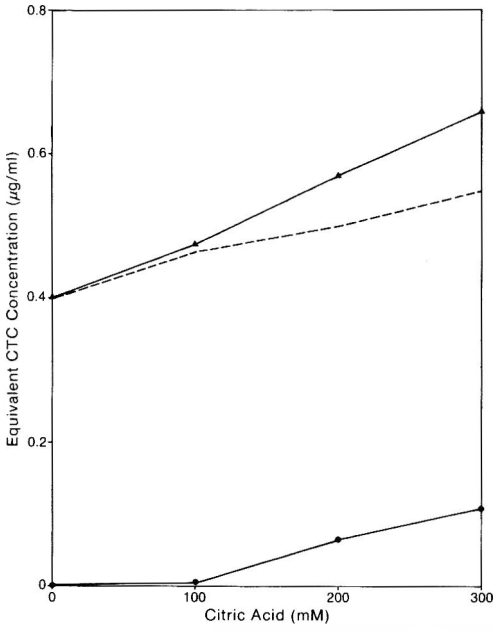

Figure 2. Enhancement of CTC activity by citric acid. Bottom line (●-●-●) represents citric acid alone; upper line (▲-▲-▲) represents citric acid plus CTC (0.4 μg/mL); dotted line is normalized line depicting CTC enhancement by citric acid.

growth rate caused by citric acid, because the normalized curve in Figure 2 should correct for such an effect. One might envision that the magnesium ions inside the cell form complexes with CTC molecules in an equilibrium state. If some of the magnesium ions are being chelated by citric acid, the equilibrium may be shifted toward a reduction in complex formation, thereby resulting in more free CTC molecules available for inhibiting protein synthesis. This is based on the assumption that the binding constant between citric acid and magnesium ions is higher than that between CTC and magnesium ions.

Since citric acid can reverse the inhibition of CTC by magnesium, the binding of magnesium to CTC must be a reversible process. Once the feed is inside the animal, the magnesium concentration will be diluted considerably; therefore, the authors do not believe that the magnesium ions are actually inhibiting the desired effect of CTC in the feed.

The citric acid results reported in this paper not only strengthen the proposed mechanism by which magnesium inhibits CTC activity (4), but they also provide a possible solution to overcome the problem of magnesium interference in CTC assay by turbidimetric method. At this stage we do not know if citric acid would improve the assay result of a particular feed sample containing high magnesium because there may be other ions that could react with citric acid before magnesium ions do. Therefore, even if these experiments were repeated with feed samples, it is difficult to draw a general conclusion on all feed samples simply because different feeds may contain different amounts of other ions. The technique could be especially useful with those feed samples that have unusually high magnesium content. Further investigation on the application of these effects on feed samples by turbidimetric method is under way in this laboratory.

Acknowledgments

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Microbiological Determination of Lincomycin in Feeds and Supplements Containing High Concentrations of Bentonite

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A microbiological method is described for determining lincomycin in complete feeds, supplements, and pre-mixes greater than 100 g/ton and containing high levels of bentonite. The AOAC method currently used is unsatisfactory for analyzing feeds with concentrations of bentonite greater than 5.5%. Study indicates that low lincomycin recoveries from high level bentonite feeds are a function of aqueous contact and subsequent binding. The present method involves an alternative extraction technique using formamide as the primary extractant. A binary solvent system of ethanol and phosphate buffer aids in the extraction, miscibility, and conversion to water solubility for subsequent testing. Forty-one feeds containing as high as 60% bentonite were assayed by the reported method and gave a mean recovery of 106% and a range of 94-114%. An inter-laboratory confirmation study produced a mean recovery of 103%, with a range of 93-114%. A factorial analysis of variance of the interactive effects between lincomycin level and bentonite level within the AOAC method and within the bentonite method showed no interactions which would influence percent recovery in either assay method.

Current AOAC methods for determining lincomycin in feeds (1, 2) are unable to analyze the antibiotic accurately in the presence of high concentrations of bentonite. Commercial supplement assays and laboratory experimentation indicated that the acid-methanol extraction method only recovers from 38 to 64% of the lincomycin from feeds containing 28-60% bentonite. Feeds containing greater than 60% have not been encountered or laboratory tested. Swine feed supplements containing large amounts of bentonite have increased and prompted the need for development of a new selective analytical technique. The method reported here involves incorporating changes in the extraction phase only of the microbiological cylinder-plate methods. The method is applicable to 100 g/ton or greater in complete feeds, supplements, and pre-mixes containing up to 60% bentonite. High levels of bentonite normally occur only in supplements containing 1000 g lincomycin or more/ton; however, 100 g/ton feeds were tested to cover unusual circumstances and to determine range limitations of the assay. The method involves primary shake extraction with formamide and subsequent combination of the fluid extract with an equal volume of a binary solvent system of ethanol and phosphate buffer. This secondary extraction phase aids in miscibility and conversion to water solubility for subsequent plate testing. A series of 4 supportive experiments were undertaken as a result of initial laboratory experimentation and problems with assay of commercial feeds routinely sent to our analytical service laboratory.

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Experimental

Materials

(a) *Laboratory preparations of feeds and supplements.*—All were typical complete swine rations ground in a Wiley mill through a 1 mm screen. The following swine feeds, S-581 and S-810, were used for laboratory preparation:

Ingredient	*Percent*
S-581:	
Ground corn	77.35
Soybean meal	19.0
Salt	0.5
Defluorinated phosphate	1.9
Fat, stabilized	1.0
Vitamins and minerals	0.2
Selenium	0.05
	100.00
S-810:	
Ground corn	78.000
Soybean meal	19.0
Dyna K	0.1
Swine pre-mix 5S	0.25
Dicalcium phosphate	1.5
Calcium	0.75
Salt	0.4
	100.00

(b) *Bentonite (Volclay sodium).*—Obtained from American Colloid Co., Skokie, IL.

(c) *Formamide (HCONH).*—98%, obtained from Aldrich Chemical Co., Milwaukee, WI 53233.

Tests

Three 100 g/ton complete feeds containing 10, 25, and 50% bentonite were assayed by the

Table 1. Three initial laboratory feed preparations and 5 routine commercial feeds assayed by both methods

Source	Lincomycin, g/ton	Bentonite in feed, %	Found, % of label: AOAC method	Found, % of label: Bentonite method
Laboratory	100	50	38, 47	97, 98
	100	25	56, 58	106, 98
	100	10	79, 82	107, 106
Com. 1 [a]	2000	40	49	100
Com. 2	2600	28	60	94
Com. 3	2000	59	50	97
Com. 4	2000	50	64	106
Com. 5	2000	59	62	106

[a] Feeds from different commercial sources, containing 28–59% bentonite.

AOAC and bentonite methods. These laboratory preparations were necessary because assays from commercial supplements showed that a problem existed with interfering bentonite concentrations.

To estimate the percent bentonite at which the AOAC and bentonite methods give different results, 10 feeds were prepared to conform to a 2 × 5 factorial arrangement of 200 and 2000 g lincomycin/ton and 0, 5, 10, 20, and 40% bentonite. All 10 feeds were assayed by both methods. Analysis of variance was done to investigate the interactive effects between level of lincomycin and bentonite concentration on the percent recovery of lincomycin.

To refine and reproduce the highest concentration of bentonite allowing accurate assay by the AOAC method, 5 feeds were prepared at 2000 g lincomycin/ton with bentonite concentrations of 0, 1.5, 3.0, 4.5, and 6.0%. Feeds were assayed by both methods.

To determine formamide toxicity influence on the results of the bentonite method, one feed at 200 g/ton was assayed at 6 different fluid extract dilutions giving final formamide concentrations of 1, 1.5, 2, 2.5, 3, and 3.5%. The 6 preparations were assayed, and percent recovery was used as the parameter for test organism toxicity. Values significantly above the 106% overall assay mean were interpreted as indicating microorganism destruction with resulting positive assay bias.

Two complete feeds, preparations 15 and 16 at 40 g/ton and 100 g/ton, respectively, were prepared from 2 different 2000 g/ton high level bentonite supplements and assayed by the AOAC method. These supplements assayed 50% and 53%, respectively. Method of preparation for complete feed 15 was one part laboratory preparation 10, 2000 g/ton, plus 49 parts feed S-581. Preparation of complete feed 16 was one part Company C supplement, 2000 g/ton, plus 19 parts feed S-581.

METHODS

Bentonite extraction procedure for feeds ≥ 100 g/ton.—Accurately weigh 4–12 g ground sample, transfer to 250 mL centrifuge bottle, add 50 mL formamide, and shake 20 min on mechanical shaker. Add 50 mL pH 8 phosphate buffer-ethanol (50 + 50). Shake 10 min, allowing solutions to equilibrate. Centrifuge 3–5 min at ca 2500 rpm. Dilute 1 mL fluid extract with pH 8 buffer to 0.33–0.88 μg lincomycin/mL.

AOAC extraction procedure.—See **42.243**(b) (3).

Assay and interpretation.—Proceed as in **42.241**, **42.242**, **42.244** (3).

Results and Discussion

The problem was demonstrated by initial experimentation with three 100 g/ton feeds containing 10, 25, and 50% bentonite assayed by the AOAC and the bentonite methods. In addition to these results, commercial supplements chronologically listed also supported the fact that the AOAC method was unsatisfactory and the bentonite method was satisfactory (Table 1).

Overall results from the first experiment support the hypothesis that when using the AOAC method, recovery of lincomycin decreases as the bentonite concentration increases. The bentonite method again produced satisfactory results irrespective of bentonite concentration (Table 2).

Analysis of the slopes of percent recovery from the 2 methods and the point of intercept between the upper 95% confidence limit on individual assays (CLI) of the AOAC method and the lower 95% CLI of the bentonite assay indicates that ca 6% bentonite is the maximum concentration that

Table 2. Estimation of % bentonite at which the AOAC and bentonite methods give different results

Feed prepn	Bentonite, %	Recovery,[a] % AOAC method	Recovery,[a] % Bentonite method
	200 g Lincomycin/ton		
1	0	106	108
		116	112
2	5	101	114
		99	106
3	10	76	114
		87	110
4	20	62	110
		63	108
5	40	48	98
		53	110
	2000 g Lincomycin/ton		
6	0	112	106
		106	104
7	5	99	108
		93	102
8	10	84	112
		88	110
9	20	67	110
		67	104
10	40	51	104
		56	102

[a] Assays of separate samples on same day.

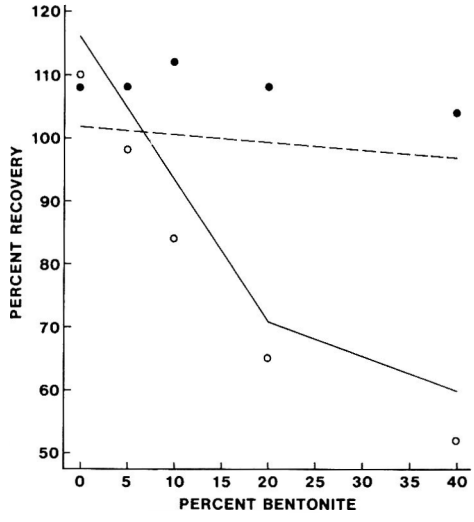

Figure 1. Analysis of slopes, first experiment. O, AOAC method; — upper 95% CLI of AOAC method; ●, bentonite method; - - lower 95% CLI of bentonite method.

can be present for accurate assay by the AOAC method (Figure 1).

The analysis of variance study for interactive effects shown in Table 3 indicates that the interaction between assay values for the levels of bentonite is the same for 200 g/ton and 2000 g/ton of lincomycin, indicating no interaction of lincomycin and bentonite levels. In addition, mean assay values for the AOAC and bentonite methods have the same relationship in 200 g/ton as in 2000 g/ton, and the trend in assay values for varying percents of bentonite and the 2 methods of assay have the same interrelationship in 200 g/ton as in 2000 g/ton. The experimental estimation of interfering bentonite concentration is corroborated in the second experiment (Table 4). The 5.5% concentration of bentonite proved to be the 100% recovery level as shown by the point of separation between the upper 95% CLI of the AOAC method and the lower 95% CLI of the bentonite method (Figure 2). Percent recovery of lincomycin from varying concentrations of formamide in the third experiment demonstrates the toxicity bias that could be effected if levels were too high (Table 5). Figure 3 shows the lincomycin recovery values with the upper and lower 95% confidence intervals on the best fitting quadratic equation. Using the technique of

Table 3. Analysis of variance for investigation of interactive effects of level of lincomycin on relationship between % bentonite and % recovery

Code	Source	DF	MS	Probability of chance diff.
Linco.	level of lincomycin	1	6.4	0.55
Ben.	% bentonite	4	1254.7	0.00
Methods	AOAC vs bentonite	1	6708.1	0.00
	Linco. × Ben.	4	17.46	0.42[a]
	Linco. × methods	1	40.0	0.14[b]
	Ben. × methods	4	1017.7	0.00
	Linco. × Ben. × methods	4	2.3	0.97[c]
	error	20	17.3	

[a] No interaction between lincomycin and level of bentonite.
[b] No interaction of lincomycin level and method.
[c] No interaction of lincomycin level, percent bentonite, and method.

Table 4. Breakpoint determination of bentonite concentration (2000 g lincomycin/ton)

Feed prepn	Bentonite, %	Recovery, % AOAC method	Recovery, % Bentonite method
6	0	108	108
		112	107
11	1.5	102	107
		114	108
12	3.0	104	107
		100	102
13	4.5	97	104
		93	108
14	6.0	86	107
		86	104

Table 5. Formamide toxicity influence on bentonite method (feed preparation 1; 200 g lincomycin/ton)

Ext diln	Formamide, %	Linco. rec., %
1:50	1.0	107
		107
1:33.3	1.5	109
		104
1:25	2.0	110
		106
1:20	2.5	126
		120
1:16.7	3.0	136
		136
1:14.3	3.5	149
		149

overlapping confidence intervals, formamide in concentrations of 2% or less can be used without altering the standard response diluent because the non-overlap of confidence intervals occurs at greater than 2% formamide. Maximum concentration of formamide attained in the experimental systems described in this report was 1.25% at the level of 100 g lincomycin/ton where the solvent system was diluted 1:40 with buffer.

In the fourth experiment, complete feeds prepared from high level bentonite supplements yielded excellent lincomycin recoveries by the AOAC method even though the supplements from which they were prepared only assayed in the 50% range by the same method (Table 6). These results are predictable in one respect because from previous experimentation the bentonite concentration is diluted to assayable levels in the complete feed, while also showing that the lincomycin in the dry high level bentonite supplement is not bound. This experiment strongly supports the fact that low lincomycin recoveries from high level bentonite feeds are a function of aqueous contact occurring in the AOAC extraction method and causing subsequent binding.

The 41 total samples assayed by the bentonite method in this report had a mean recovery of 106% with a range of 94–114%. The results of samples analyzed collaboratively by the Clinical Research Laboratories (CRL), The Upjohn Co., using the bentonite method, had a mean recovery of 103% with a range of 93–114%. Their results on the same samples assayed by the AOAC method also closely reproduced those of our laboratory, Analytical Chemical Services (ACS) (Table 7). The pre-mix used for preparation of the feeds in this study assayed at 107% which is

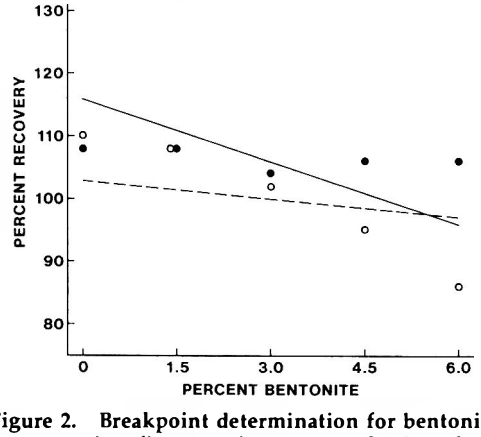

Figure 2. Breakpoint determination for bentonite concentration, first experiment: ○, AOAC method; — upper 95% CLI of AOAC method; ●, bentonite method; – – lower 95% CLI of bentonite method.

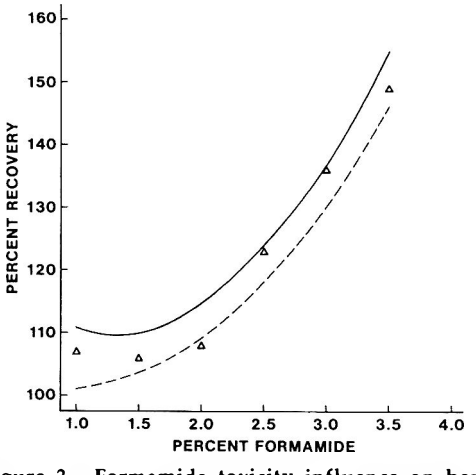

Figure 3. Formamide toxicity influence on bentonite method, third experiment: —, upper 95% confidence limit on mean of 2 assays (CLM); △, bentonite method data points; – –, lower 95% CLM.

Table 6. Complete feeds prepared from high level bentonite supplements and assayed by AOAC method

Feed prepn	Linco., g/ton	Supplement source	% Recovery, supplements	% Bentonite in comp. feed	% Recovery, complete feeds
15	40	Lab. prepn 10, 40% bentonite	53	0.8	107 111
16	100	Company C, 59% bentonite	50	2.95	106 103

Table 7. Results of duplicate samples collaboratively studied

Feed prepn	Linco., g/ton	Bentonite, %	Recovery, %			
			AOAC method		Bentonite method	
			ACS	CRL	ACS	CRL
5	200	40	48, 53	49, 58	98, 110	95, 110
10	2000	40	51, 56	64, 64	104, 102	95, 111
6	2000	0	112, 106	107, 107	106, 104	98, 114
9	2000	20	67, 67	65, 65	110, 104	98, 101

responsible for an apparent positive bias over an expected 100% recovery. This fact, however, is insignificant inasmuch as the influence is the same for both methods. The manufacturer puts a 5% flush in the pre-mix to assure customer receipt of 100% of the product. A limitation found accentuating 100 g/ton as the lower limit of sensitivity for this described technique is the fact that sample size cannot exceed 25% of the bentonite extract solvent volume when feed samples contain 50% or more bentonite. Sample sizes greater than this produce a mixture that hardens completely and cannot be processed.

Chemical properties relative to the extraction capability of formamide include miscibility with water and alcohols, and ability to dissolve substances such as casein, lignin, cellulose, other proteinaceous material, and synthetics such as nylon. It is a good softener for water-soluble gums and animal glues (4). The amide linkage in the solvent makes it especially compatible with organic materials such as feeds (5). Use of formamide as an extract solvent in feeds could be a valued addition to feed analysis for other antibiotics as well as lincomycin and should be tried where synthetics, pelletizing agents, lignin, or other adhesive ingredients are suspected problems. The bentonite method described was designed to support conditions relative to known and expected usage of bentonite. Technical flexibility does exist for modifying this method to apply to other situations as they arise, but at this time it does not appear to be necessary and the method described should facilitate rapid acceptance and implementation.

Acknowledgments

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High Pressure Liquid Chromatographic Determination of Carbadox and Pyrantel Tartrate in Swine Feed and Supplements

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A rapid yet reliable procedure for the simultaneous extraction and assay of carbadox and pyrantel tartrate is described. The feed is extracted with water-acetonitrile-methanol and cleaned up on a short alumina column. The eluant is separated by high pressure liquid chromatography and the compounds are detected at different wavelengths. The drugs of interest are well resolved in all feeds studied. The procedure has also been applied to a wide range of feeds which contained either one of the drugs or both in combination. No significant interferences were observed. Spiked sample recoveries were 97% for carbadox and 101% for pyrantel tartrate. Ruggedness test coefficients of variation were 2.0% for carbadox and 2.1% for pyrantel tartrate.

Carbadox, an antimicrobial agent, and pyrantel tartrate, an anthelmintic, can be incorporated into swine feed either together or separately in combination with other drugs. The most frequent use appears to be a combination at 0.0055% carbadox and 0.0106% pyrantel tartrate (finished feed) or 0.055% carbadox and 0.106% pyrantel tartrate (supplement). There are published methods for the spectrophotometric determination of both carbadox (1–4) and pyrantel tartrate (5, 6) as well as high pressure liquid chromatographic (HPLC) methods for carbadox (7, 8) and pyrantel tartrate (9). However, none of these procedures lends itself to a concurrent analysis of these 2 drugs.

The procedure presented here provides such an analysis with the added advantage that it does not require the method of standard addition used by Goras et al. (1, 4) for carbadox or by Litchman (6) for pyrantel tartrate.

METHOD

Caution: Solutions of both carbadox and pyrantel tartrate are light-sensitive. Protect both feed extracts and standard solutions from sun or fluorescent light.

Apparatus

(a) *High pressure liquid chromatograph.*—Two Model 6000A pumps, Model 730 system controller, Model 720 data module, Model 710B Wisp autosampler, and Model 440 dual channel spectrophotometric detector with 313 and 365 nm filters (Waters Associates, Milford, MA 01757), or equivalent.

(b) *HPLC columns.*—Waters radial compression module with 5 mm id × 10 cm C18 cartridge and Waters 84550 guard column containing 30–40 μm pellicular C18 packing.

Reagents

(a) *Extractant.*—Methanol–acetonitrile (1 + 1), both HPLC grade.

(b) *Alumina.*—Fisher neutral, A-950. Stir 200 g alumina with 1 L water for 30 min. Pour off fines, resuspend, and filter through glass fiber filter (Whatman GFA) in Buchner funnel. Dry with vacuum and wash 3 times with methanol. Dry in forced air oven at 80°C overnight and store in desiccator.

(c) *Mobile phase.*—18–20% HPLC grade acetonitrile and 82–80% dibutylamine acetate (DBAA) solution at flow rate of 2 mL/min. Prepare DBAA stock solution by titrating dibutylamine (DBA) (Fisher 0-2199) with acetic acid to pH 2.5 (100 mL DBA and ca 270 mL acetic acid). Dilute 25 mL stock solution to 1 L with water to produce working solution. Filter this solution through 0.45 μm glass fiber filter and degas before using. Working solution is 0.04M DBAA with pH of 3.7.

Standard Solutions

Store all solutions in freezer in low actinic glassware. Both carbadox and pyrantel tartrate reference standards are available from Pfizer, Inc., Lee's Summit, MO 64063.

(a) *Carbadox stock solution.*—550 μg/mL. Weigh 0.055 g carbadox into 100 mL volumetric flask. Dissolve and dilute to volume with $CHCl_3$–methanol (3 + 1).

(b) *Carbadox intermediate solution.*—55 μg/mL. Dilute 10 mL stock solution to 100 mL with extractant.

(c) *Pyrantel tartrate stock solution.*—1.06 mg/mL. Weigh 0.106 g pyrantel tartrate into 100 mL

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volumetric flask. Dissolve and dilute to volume with extractant.

(**d**) *Pyrantel tartrate intermediate solution.*—106 μg/mL. Dilute 10 mL stock solution to 100 mL with extractant.

(**e**) *Mixed carbadox and pyrantel tartrate working solution.*—11 μg carbadox/mL and 21.2 μg pyrantel tartrate/mL. Pipet 20 mL of both intermediate solutions into same 100 mL volumetric flask, add 30 mL water, and dilute to volume with extractant.

Preparation of Samples

Grind feed to pass 20 mesh sieve. Into 125 mL screw-cap Erlenmeyer flask, weigh sample portion to contain 0.55 mg carbadox and/or 1.06 mg pyrantel tartrate. Wet samples with 15 mL water, mix well, and let equilibrate 5 min. Add 35 mL extractant, cap, and shake vigorously 30 min. Centrifuge or filter mixture through glass fiber filter (Whatman GFA). Prepare cleanup column by dry-packing 4 g alumina into 1 cm column with sintered glass plug, and add 15 mL sample extract. Collect only first 4 mL of eluate.

Determination

Inject 25 μL mixed standard and adjust flow rate and mobile phase concentration so that retention volumes are 1.3 for carbadox and 2.5 for pyrantel tartrate. Switch detector from 365 nm at 0.1 AUFS to 313 nm at 0.05 AUFS after carbadox has been completely eluted. Inject 25 μL of sample under same conditions. Inject standard after every second sample and compare peak area of standard (S) with peak area of sample (X).

$$\% \text{ Carbadox} = (X)(0.055)/(S)(\text{sample wt})$$

$$\% \text{ Pyrantel tartrate} = (X)(0.106)/(S)(\text{sample wt})$$

Discussion

Some C18 HPLC columns contain unreacted silanol sites which may cause tailing or, in extreme cases, absolute retention of the pyrantel tartrate. The addition of DBAA to the mobile phase eliminates much of this problem. On columns with few uncapped silanols the order of elution may be reversed. Both a Waters μBondapak C18 and a Waters RCM-Z C18 column exhibited this phenomenon with a mobile phase of 18% acetonitrile and 82% DBAA solution at a flow rate of 2 mL/min. Retention times were 2.4 min for pyrantel tartrate and 3.5 min for carbadox. An IBM stainless steel column (45 mm id × 250 mm) with 5 μm spherical C18 packing required 80% DBAA and 20% acetonitrile at a flow rate of 2 mL/min. Retention times were 2.62 min for carbadox and 3.52 min for pyrantel tartrate.

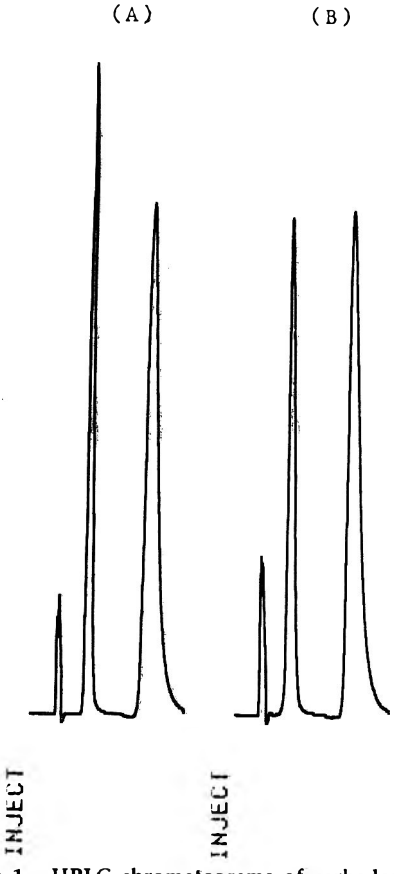

Figure 1. HPLC chromatograms of carbadox and pyrantel tartrate standard solutions with (A) and without (B) added water.

On the columns that have been tried, an increase in the percentage of acetonitrile in the mobile phase caused uniform decreases in retention volumes for both drugs. Increasing the molarity of the DBAA solution decreased the retention volume of pyrantel tartrate with little effect on carbadox. Decreasing the molarity of the DBAA increased the retention volume of pyrantel tartrate with little effect on carbadox. However, this decrease in molarity causes the pyrantel tartrate peak to tail.

Figure 1 shows 2 chromatograms from the RCM C18 cartridge. The first is of a standard solution, and the second is of the same standard without the 30% added water. Retention times are the same for both, peak areas are the same, and pyrantel tartrate peak heights are the same.

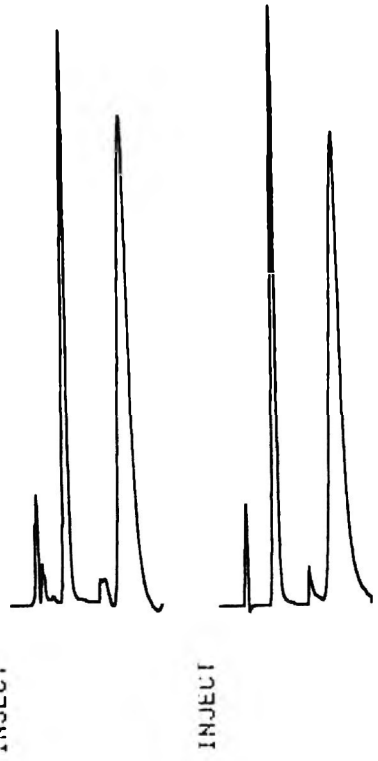

Figure 2. HPLC chromatograms of 2 typical feeds both guaranteed to contain 0.0055% carbadox and 0.0106% carbadox.

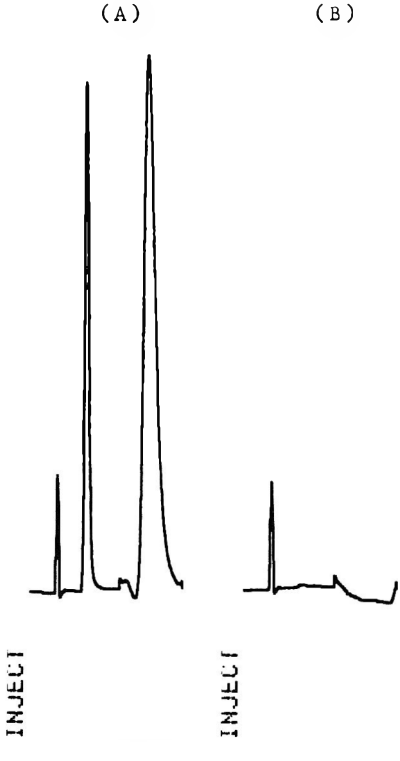

Figure 3. HPLC chromatograms of (A) feed spiked at 0.0055% carbadox and 0.0106% pyrantel tartrate and (B) the same feed unspiked.

The peak height for carbadox is considerably lower when analyzed without added water.

This procedure was developed in the fall and winter of 1980 and has been used as a screen in this laboratory since that time. Characteristic chromatograms of samples analyzed are shown in Figure 2. The shift in baseline between the 2 peaks is caused when switching from one detector at 365 nm to another at 313 nm. The absence of interference is typical of the samples analyzed.

Figure 3 shows chromatograms of an unspiked and a spiked feed. Again the baseline is clean, showing little interference. The spike recovery was 96% for carbadox and 99% for pyrantel tartrate.

The average spiked sample recovery for carbadox was 97%. This procedure was also compared to the official spectrophotometric method (10). A series of 21 routine regulatory samples with guarantees ranging from 0.00375 to 0.055% carbadox was analyzed. These results were subjected to a paired-*t* statistical test. The test statistic for 20 degrees of freedom was 0.15 which is well below 2.09 (the critical value of *t* at the 5% level). This indicates that there is no difference in results between the 2 methods.

Comparison assays were made on pyrantel tartrate using the method described in this paper and the Goras HPLC procedure (9). Spike recovery is excellent by both methods, 99% by the Goras procedure and 101% by the present method. However, actual feed and supplement analyses indicate a problem. Routine samples analyzed by both methods frequently gave higher results by the present procedure.

Table 1. Comparison of pyrantel tartrate results (%) by different HPLC procedures

Lot	Pfizer (Goras procedure)[a]	NCDA (Goras procedure)[b]	NCDA (this procedure)[b]
1	0.107	0.106	0.126
2	0.102	0.102	0.128
3	0.105	0.105	0.122
4	0.0115	0.0114	0.0123
5	0.0116	0.0116	0.0114
6	0.0116	0.0116	0.0123

[a] Each value is the average of 6–10 determinations.
[b] Each value is the average of 3 determinations.

Table 2. Effect of slight variations in conditions on determination of carbadox and pyrantel tartrate in swine feed[a]

		Carbadox		Pyrantel tartrate	
Condition	Variations	Found, %	Diff.	Found, %	Diff.
Amount of light	very dark	0.00572		0.01110	
	moderately dark	0.00562	0.00010	0.01089	0.00022
Filter	Whatman GFA	0.00574		0.01107	
	Reeve Angel	0.00561	0.00013	0.01092	0.00015
Shaking time	30 min	0.00567		0.01093	
	20 min	0.00567	0.00000	0.01106	−0.00014
Sample type	dense	0.00567		0.01096	
	voluminous	0.00567	0.00000	0.01103	−0.00007
Wt alumina	4.0 g	0.00571		0.01108	
	3.7 g	0.00564	0.00007	0.01091	0.00017
HPLC column	RCM C18	0.00569		0.01110	
	RCM-Z C18	0.00565	0.00004	0.01089	0.00020
Equilibration time	5 min	0.00572		0.01108	
	10 min	0.00562	0.00010	0.01091	0.00017

[a] Commercial feed guaranteed at 0.0055% carbadox and 0.0106% pyrantel tartrate.

Therefore, split samples were obtained from Pfizer. These were analyzed at Pfizer by the Goras procedure (9) and in this laboratory by both procedures. The results in Table 1 indicate that the aberration is more apparent for supplements than for finished feed. Further work is needed to resolve the discrepancies between the 2 procedures.

This procedure has been subjected to a ruggedness test to determine the effects of slight changes in sample, extraction, and chromatographic conditions. A commercial swine feed guaranteed to contain 0.0055% carbadox and 0.0106% pyrantel tartrate was analyzed under varying experimental conditions (Table 2). The standard condition specified all fluorescent lights out and window blinds closed (very dark). The light was then varied by (*1*) allowing some fluorescent light from a side work bench but keeping the overhead lights off and the blinds shut (moderately dark), and (2) opening the blinds and turning on the overhead fluorescent lights. The second set of conditions caused tremendous changes; the carbadox result was 28% lower and erratic and the pyrantel tartrate result was 74% lower and more erratic. Pyrantel tartrate results were further compromised by the co-elution of 2 other degradation products which were totally absent under low light conditions.

Even with a small increase in light, the pyrantel tartrate results were depressed enough to make this change the most significant. Carbadox was not affected to the same degree.

No other significant sensitivity to slight change was noted. Condition (*1*) coefficients of variation were 1.97% for carbadox and 2.13% for pyrantel tartrate.

These low coefficients of variation indicate that this procedure can be used in other laboratories to produce similar results on both drugs. Also, the carbadox comparison study indicates no observable difference between this method and the official spectrophotometric method (10). However, comparison of pyrantel tartrate results between this method and the Goras procedure (9) indicate a bias in at least one of the methods. This problem should be studied further.

Acknowledgment

The authors thank R. L. Smallidge, Department of Biochemistry, Purdue University, for an alumina column modification used in this study.

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COFFEE AND TEA

High Performance Liquid Chromatographic Determination of Caffeine in Decaffeinated Coffee, Tea, and Beverage Products

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A method was developed for determining caffeine in decaffeinated coffee, tea, and beverage products by high performance liquid chromatography (HPLC). The HPLC system consisted of a Bio-Sil ODS-5S C_{18} column, methanol-water (25 + 75) mobile phase at 1 mL/min, and a UV detector. The method is simple and specific. Caffeine recoveries were 93.8-98.3% and coefficients of variation were 0.90-2.25%.

Concern about the possible adverse health effects of caffeine on the consumer has resulted in the development of decaffeinated coffee, tea, and beverage products. These decaffeinated products are gaining more acceptability by the consumer and their number in the market is gradually increasing.

Decaffeinated food products contain approximately 1-3% of the original amount of caffeine present in the parent products. Because of their much lower caffeine content, caffeine in decaffeinated food products cannot be determined reliably by the methods developed for food products with full caffeine content (1-3), and should be determined by methods developed specifically for such products.

An official AOAC method exists for the determination of caffeine in decaffeinated coffee only (4). Other methods for the determination of caffeine in decaffeinated coffee have also been reported (3, 5, 6). However, there are no methods for the reliable determination of caffeine in decaffeinated tea or beverage products.

The objective of this study was to develop a versatile and accurate high performance liquid chromatographic (HPLC) method for the determination of caffeine in all these products.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Waters Associates equipped with Model 6000A pump, Model U6K injector, and a data module (Waters Associates, Milford, MA 01757).

(b) *Detector.*—Gilson Model 222 (Gilson Medical Electronics, Middleton, WI 53562), set at 277 nm and sensitivity unit of 0.02.

(c) *HPLC column.*—250 × 4 mm Bio-Sil ODS-5S (Bio-Rad Laboratories, Richmond, CA 94804).

(d) *Rotary evaporator.*—Buchi Model R110 (Brinkmann Instruments, Inc., Westbury, NY 11590).

(e) *pH meter.*—Corning Model 10 (Corning Scientific Instruments, Medfield, MA 02052).

(f) *Ultrasonic bath.*—Model ME 5.5 (Mettler Electronics, Anaheim, CA 92803).

(g) *Vortex mixer.*—Model K-550-G (VWR Scientific, San Francisco, CA 94119).

Reagents

(a) *Caffeine standard solution.*—20 μg caffeine/mL. Accurately weigh 20 mg caffeine (USP, anhydrous, Sigma Chemical Co., St. Louis, MO 63178) into 100 mL volumetric flask, dissolve, and dilute to volume with HPLC water. Dilute 10 mL aliquot of this solution to 100 mL with HPLC water.

(b) *HPLC mobile phase.*—Methanol (chromatography grade)-HPLC water (25 + 75).

(c) *HPLC water.*—Pass distilled, deionized water through 0.45 μm filter membrane. Use throughout method.

Extraction of Caffeine

(a) *Decaffeinated ground coffee.*—Accurately weigh ca 1 g into 250 mL beaker, add ca 100 mL water, and heat with stirring at ca 90°C for 20 min. Cool to room temperature and filter through Whatman No. 1 paper. Adjust pH of filtrate to 10 with 5% NaOH solution and transfer to 500 mL separatory funnel. Extract 3 times with twice the volume of $CHCl_3$. Combine $CHCl_3$ extracts and pass through anhydrous Na_2SO_4. Reduce volume of filtrate to ca 10 mL

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in rotary evaporator; then transfer quantitatively to test tube and evaporate to dryness under nitrogen. Add 5 mL hot water (ca 90°C) to residue in test tube, mix thoroughly with vortex mixer, and transfer washing to 50 mL beaker. Repeat water extractions 4 times, and cool washings to room temperature. Filter washings through 0.45 μm membrane filter and transfer to 25 mL volumetric flask. Dilute to volume with water and store for HPLC analysis.

(b) *Decaffeinated instant coffee.*—Proceed as in (a), except use 0.5 g sample and heat with hot water for only 10 min.

(c) *Decaffeinated leaf tea.*—Proceed as in (a).

(d) *Decaffeinated beverages.*—Decarbonate 100 mL in ultrasonic bath for 5 min. Extract with $CHCl_3$ and proceed as in (a), except wash final residue 3 times with 3 mL hot water, and dilute final volume of filtrate to 10 mL in 10 mL volumetric flask.

Figure 1. Chromatogram of standard caffeine (76 ng).

HPLC Analysis

Inject 10 μL caffeine standard solution into liquid chromatograph. Establish retention time and peak area of the caffeine peak and calibrate data module accordingly (external standard method). Inject 10–20 μL of sample filtrates and obtain amount of injected caffeine directly from calibrated data module. Calculate caffeine content in mg/g for solid samples and in mg/100 mL for liquid samples. Re-inject 10 μL caffeine standard solution and recheck data module calibration at regular intervals.

Recovery Study

Add the proper volume of the caffeine standard solution to each sample so that caffeine content of spiked sample will approximately double. Determine caffeine content of spiked sample as described previously and calculate percent recovery.

Results and Discussion

To extract the maximum amount of caffeine from decaffeinated products, we investigated the effect of heating time with water and the effect of the pH of the sample aqueous extract before $CHCl_3$ extraction. Several heating times ranging from 5 to 30 min were tried and caffeine content was determined by the HPLC method. The maximum amount of caffeine was extracted from decaffeinated instant coffee after heating with water for 10 min at about 90°C. Both decaffeinated ground coffee and leaf tea samples required a heating time of 20 min for maximum caffeine yield. Heating in a dilute NH_4OH solution instead of water did not increase the amount of caffeine extracted from any of the samples. The pH value of the aqueous extracts was adjusted to range from 3 to 10 before $CHCl_3$ extraction. In all samples analyzed, aqueous extracts with pH of 10 yielded the maximum caffeine content. It was also important to extract aqueous extracts with twice the volume of $CHCl_3$ to avoid the formation of an emulsion.

A calibration curve was constructed to investigate the linearity of the detector response to various caffeine concentrations. A linear response was obtained over a range of 10–500 ng caffeine. The results indicated also that the HPLC method is sensitive; caffeine could be determined reliably at ng levels (Figure 1).

Under the HPLC conditions used in this study, caffeine was separated as a single peak with a retention time of 12.8 min. The caffeine peak was well resolved with no interference in any chromatogram obtained. As an example, typical chromatograms of decaffeinated instant coffee and tea samples are shown in Figure 2.

The caffeine content of 4 samples of decaffeinated ground coffee is presented in Table 1. In general, the caffeine content of decaffeinated instant and ground coffee obtained by the HPLC method was lower than that reported in previous studies (3, 5, 6). This is probably due to differences in brands and also in the methods used for caffeine analysis. The chromatographic-spectral methods used for caffeine determination in decaffeinated coffee (3, 6) are less specific because

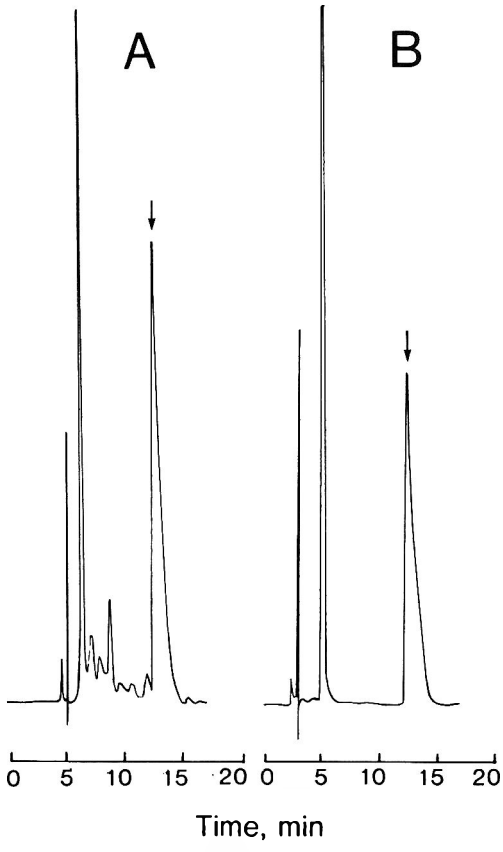

Figure 2. Chromatograms of decaffeinated coffee (A) and decaffeinated tea (B). Caffeine peak is marked by arrow and represents 251 ng caffeine in (A) and 194 ng caffeine in (B).

Table 1. Caffeine content of decaffeinated samples

Sample	Caffeine content, mg/100 mL	CV, %
Coffee[a]		
Brand A instant	1.083 ± 0.031	2.78
Brand B instant	0.970 ± 0.012	1.03
Brand C instant	0.970 ± 0.038	3.87
Brand D instant	0.779 ± 0.027	3.49
Brand A ground	0.444 ± 0.013	2.93
Brand B ground	0.224 ± 0.007	3.30
Brand C ground	0.139 ± 0.003	2.17
Tea[a]		
Brand A	0.878 ± 0.032	3.60
Brand B	0.822 ± 0.013	1.61
Brand C	0.647 ± 0.018	2.73
Brand D	0.593 ± 0.016	2.67
Beverages[b]		
Brand A (cola)	0.0146 ± 0.0001	0.82
Brand B (cola)	0.0426 ± 0.0023	4.65
Brand C (root beer)	0.3660 ± 0.0065	1.78

[a] Average of 6 determinations.
[b] Average of 3 determinations.

Table 2. Recovery of caffeine from various decaffeinated products

Product	Recovery, %[a]	CV, %
Brand A instant coffee	98.3 ± 1.9	1.93
Brand A ground coffee	98.3 ± 2.2	2.25
Brand A tea	95.2 ± 0.98	1.03
Brand C root beer	93.8 ± 0.85	0.90

[a] Average of 6 determinations.

the final sample extract may contain interfering compounds which absorb at the same wavelength as caffeine and contribute to higher caffeine contents. In the HPLC method reported by Smyly et al. (5) for the determination of caffeine in decaffeinated instant coffee, the sample was solubilized in water and the aqueous extract was injected directly into the liquid chromatograph. However, preliminary results obtained at the early stages of this study indicated that $CHCl_3$ extraction of the aqueous extract of any of the decaffeinated coffee samples minimizes interference, improves the resolution of the caffeine peak, and yields more accurate caffeine contents.

The caffeine contents of 4 decaffeinated tea samples and 3 decaffeinated beverages are also shown in Table 1. As far as we know, no other studies have reported the caffeine content of these samples.

The recovery of caffeine from the spiked samples obtained by the HPLC method ranged from 93.8 ± 0.85 to 98.3 ± 1.9% as shown in Table 2. These results indicate that the HPLC method has a high degree of accuracy. The method is also precise; coefficients of variation were less than 5% in all samples tested.

The AOAC method (4) was used to determine caffeine content of decaffeinated ground and instant coffee samples in order to compare it with the HPLC method. The AOAC method (4) yielded higher caffeine content than the HPLC method in both decaffeinated instant and ground coffee samples as shown in Table 3. This may be due to the fact that the AOAC method is a chromatographic-spectral method which is less specific for caffeine than the HPLC method. Some of the interfering compounds extracted with

Table 3. Caffeine determination in decaffeinated coffee by HPLC and AOAC methods

Product	Caffeine, mg/g		CV, %	
	HPLC[a]	AOAC[b]	HPLC	AOAC
Brand B instant	0.970 ± 0.012	1.123 ± 0.084	1.03	7.51
Brand B ground	0.224 ± 0.007	0.371 ± 0.015	3.30	4.04

[a] Average of 6 determinations.
[b] Average of 3 determinations.

caffeine may have contributed to the higher caffeine content obtained by the AOAC method. In the HPLC method, caffeine is determined as a single peak with no interference.

The developed HPLC method has been shown to be accurate and precise. It is more specific and more versatile than the AOAC method.

Acknowledgments

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METALS AND OTHER ELEMENTS

Hot Leaching of Ceramic and Enameled Cookware: Collaborative Study

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A hot leach method published by the World Health Organization for determining Pb and Cd in ceramic and enameled ware was collaboratively studied in 14 laboratories. The method consisted of heating a solution of 4% acetic acid at the boil for 2 h in 6 samples of specially glazed ceramic ware and 6 samples of special enameled ware. The acid was allowed to cool and stand in contact with the ware for an additional 22 h. At the end of the 2 h heating period and again at the end of the 24 h period, the leach solution was assayed by atomic absorption spectrophotometry for Pb and Cd. Ruggedness testing before the collaborative study revealed that: (*1*) the quantity of metal released increased as the average leach temperature approached the boiling point, (*2*) the quantity of Pb and Cd released from enameled ware increased linearly with time during 7 h of boiling, (*3*) the concentration of acid could be varied from 2 to 6% with no apparent effect on the amount of metal released, and (*4*) the room temperature (RT) contact period lasting from 22 to 120 h (after the 2 h heating period) did not increase the amount of metal released. The quantities of Pb and Cd measured by the collaborators at the end of 2 h of heating and after 24 h of total contact were essentially identical. The amounts of Pb and Cd were not related to the quantity of acid solution required to restore the leach solution volume to its initial value after boiling. The collaborative results showed that the period of standing at RT is not necessary and probably can be eliminated from the method. The modified method, which requires analysis of the leach solution immediately after the 2 h heating period, has been adopted interim official first action by AOAC.

The official AOAC method (1) for measuring Pb and Cd released from earthenware and ceramic ware glazes requires a simulating 4% acetic acid leaching solution and a contact time of 24 h at room temperature (RT). This method of determining the quantity of toxic metals released is employed by a number of countries (2, 3). However, the quantity of Pb and Cd extracted by acidic solvents at elevated temperatures may greatly exceed the quantity of these elements released by the AOAC-RT contact method (4–8). In addition, various foods, heated or cooked in contact with such metal-releasing surfaces, also become contaminated (9–11). For these reasons it was desirable that a hot leach method be investigated, one which would more nearly represent the metal release by ware used for cooking and baking.

Ruggedness Testing

Before the collaborative study was begun, several variables of potential importance to the validity of the analytical results were investigated. These were: (*1*) the influence of temperature near the boiling point, (*2*) the contact time at RT, (*3*) the contact time at elevated temperatures, and (*4*) the restoration of the fill volume.

Influence of Temperature Near the Boiling Point

The initial temperature used was about 60°C. This temperature was increased stepwise to the boiling point by using several samples. All contained 4% acetic acid solution.

The temperature was read 2–3 times during the 2 h heating period, and the average temperature

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The recommendations of the Associate Referee were approved by the General Referee and by Committee E. The method was adopted interim official first action following the 96th Annual International Meeting of the AOAC, Oct. 25–28, 1982. See *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.
This report of the Associate Referee, J. H. Gould, was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.
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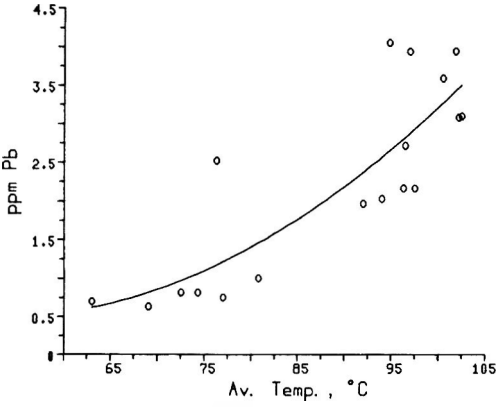

Figure 1. Influence of increasing leach temperature on release of Pb from enameled ware.

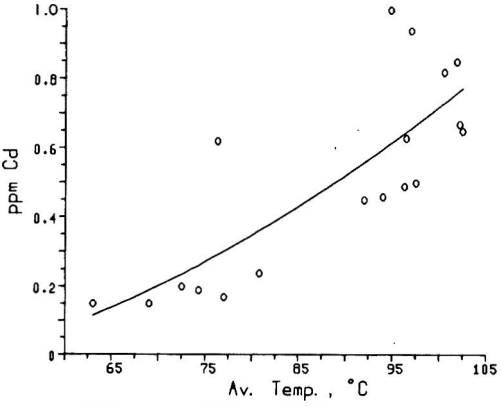

Figure 3. Influence of increasing leach temperature on release of Cd from enameled ware.

was recorded. A sample was taken at the end of 2 h, at the end of 24 h, and then daily for as long as 120 h total contact (all but the initial 2 h was at RT).

No statistical difference in the Pb or Cd concentration in the leach solution was observed between the 2 and 24 h samples. The Pb released from the enameled ware during the 24 h vs the average temperature during the 2 h heating period is given in Figure 1. The second degree equation of this regression line is:

$$\text{Pb, ppm} = 1.9371 - 0.08566T + 0.000982T^2$$

where T is the average leaching temperature in °C. A plot of the data is given in Figure 1.

The amount of Pb released from the ceramic glaze during 24 h vs the average temperature during the 2 h heating period is given inFigure 2. The second degree equation of the regression line is:

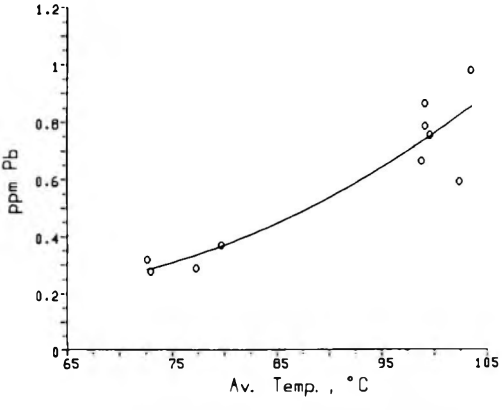

Figure 2. Influence of increasing leach temperature on release of Pb from ceramic ware.

$$\text{Pb, ppm} = 3.4150 - 0.08154T + 0.0005504T^2$$

The Cd released from enameled ware during 24 h vs the average temperature of the leach solution during the 2 h heating period is given in Figure 3. The regression line is given by the second degree equation:

$$\text{Cd, ppm} = 0.7936 - 0.02678T + 0.000255T^2$$

Contact Time at Room Temperature

The amounts of Pb and Cd released during moderate periods of continuous RT contact between enameled or ceramic ware and the 4% acetic acid solution are given in Tables 1 and 2. There was no statistically significant increase in either Pb or Cd content after the initial heating period.

Long term (151–154 days) RT contact of acetic acid with enameled ware produced 2 distinct phases, depending on the leach temperature initially employed. A range of 63–81°C (n = 5) resulted in an average Pb increase of 50.7% (standard deviation (SD) 41.7) relative to the Pb content as initially measured. An average Cd increase of 90.6% (SD 52.0) was observed relative to the initial Cd. Conversely, a leach temperature in the range of 92–98°C (n = 4) resulted in a *decrease* in Pb content of 8.2% (SD 2.3) relative to the initial quantity. The Cd content increased only 7.9% on the average (SD 2.04) for this higher temperature group.

Concentration of Acetic Acid Solution

No significant difference was observed in the quantity of metal released by the standard 4% acetic acid solution (Tables 1 and 2) relative to that released by a 2 or 6% solution (Tables 3 and

Table 1. Pb and Cd (ppm) released from enameled ware by 4% acetic acid as a function of initial heating temperature and RT contact time

Av. leach temp., °C[a]	Contact time, h				
	2[a]	24	48	72	96
			Pb		
63	0.70	0.72	0.77	0.76	0.80
69	0.64	0.69	0.80	0.80	0.77
74	0.82	0.84	0.77	0.87	
75	0.82	0.92	0.91	0.92	0.89
77	0.76	0.91	0.92	0.78	
81	1.01	1.04	1.12	1.03	
92	1.97	2.05	2.05		
94	2.04	2.18	2.14		
96	2.17	2.25	2.24		
97[b]	3.95	4.08			4.09
98[c]	3.47	3.45			3.43
			Cd		
63	0.15	0.18	0.21	0.19	0.18
69	0.15	0.19	0.20	0.18	0.17
74	0.19	0.18	0.17	0.18	
75	0.20	0.19	0.22	0.19	0.21
77	0.17	0.17	0.18	0.20	
81	0.24	0.23	0.24	0.24	
92	0.45	0.44	0.45		
94	0.46	0.45	0.46		
96	0.49	0.48	0.49		
97[b]	0.94	0.86			0.95
98[c]	0.83	0.72			0.80

[a] 2 h hot leach period.
[b] 200 mL makeup volume.
[c] 78 mL makeup volume.

4). The fact that the same amount of metal is released under various acid concentrations may be considered as an important stabilizing influence for this method, for under conditions of boiling, the acetic acid concentration of the leach solution would probably change.

Contact Time at Elevated Temperatures

In contrast to the nearly static metal content found in the acetic acid during continued RT contact, continued heating of the 4% acetic acid solution at an average temperature of about 102°C in enameled ware for 7 h resulted in a continuing increase in the concentration of Pb and Cd.

The linear regression plot of Pb concentration vs the time of heating resulted in a line having a positive slope of 1.2 ppm Pb/h and a coefficient of determination of 0.998. The release of Cd also increased linearly with the time of heating. A plot of these values gave a regression line with a positive slope of 0.3 ppm Cd/h, with a coefficient of determination of 0.997.

Table 2. Pb (ppm) released from ceramic ware by 4% acetic acid

Ware No.	Av. leach temp., °C[a]	Makeup vol., mL	Contact time, h					
			2[a]	24	48	72	96	120
1	73	15	0.28	0.38	0.45	0.27	0.32	
2	73	8	0.32	0.44	0.37	0.36		
3	77.3	14	0.29	0.37	0.45	0.30	0.38	
4	79.7	23	0.37	0.40	0.40	0.40	0.48	
5	98.7	44	0.66	0.65	0.76			0.72
6	99.0	38	0.78	0.76	0.84			0.83

[a] 2 h heating period.

Table 3. Cd and Pb (ppm) released from enameled ware by 2–6% acetic acid

Av. leach temp., °C[a]	Makeup vol., mL	Acetic acid concn, %	Contact time, h: Pb 2[a]	Pb 24	Pb 48	Cd 2[a]	Cd 24	Cd 48
102	100	2	3.10	2.85		0.67	0.63	
101	200	2	3.60	3.06		0.82	0.68	
98	190	2.4	2.17	2.19	2.30	0.50	0.47	0.49
103	100	2.4	2.92	3.11	3.04	0.65	0.61	0.65
95	0	6.0	4.06	4.27	4.18	1.00	0.93	0.99
97	0	6.0	2.72	2.90	2.91	0.63	0.60	0.58

[a] 2 h hot leach period.

After the 7 h heating period the enameled ware was allowed to cool to RT. An aliquot analyzed at 24 h showed no increases in Pb or Cd over the aliquot analyzed at the end of the 7 h heating period. Thus, the length of contact time at elevated leach temperatures is an important factor in determining the quantity of metal released. For this reason, the high temperature contact time specified by the method must be carefully followed.

Restoration of Fill Volume

One way to restore the volume of the leach solution after heating is by establishing the correct depth with a "dip-stick." One rod was prepared by placing a scratch on a glass rod corresponding to the average depth of 1120 mL RT water contained in 3 enameled pans. A second rod was prepared at the average depth of 187 mL RT water contained in 3 ceramic casseroles.

The rod in the enameled ware was tested by measuring the volume of RT water required to fill 9 enameled pans 3 times each to the mark. The average volume (n = 27) was 1112.4 mL (SD 10.3 mL). The average volume of 3 refills of 10 ceramic casseroles (n = 30) was 192.2 mL (SD 3.05 mL).

The need for volume restoration for the period of RT contact of the leach solution could be eliminated if the 2 h heating period alone is used. In that case, the volume of the test solution and its restoration can be determined by using a graduated cylinder or other direct means before assay.

Conclusions from Ruggedness Testing

The temperature at which the leaching is conducted and the time of the high temperature contact period have an important influence on the quantity of Cd and Pb released. However, the contact time at RT after the active heating phase is completed has a minimal influence on increasing the quantity of Cd and Pb released for as long as 120 h. Then the need to assay immediately does not appear to be necessary.

Collaborative Study

The ware used in this study was *not* a commercial product. The ceramic casseroles and covers had been made with a special glaze that released sufficient Pb to permit its accurate determination by atomic absorption spectrophotometry (AAS). The enamel that had been applied to the pans had also been specially prepared for this study, and it released both Pb and Cd in quantities sufficient to permit direct determination of these metals by AAS.

Six ceramic casseroles with covers and 6 enameled pans with lids were distributed to each collaborator according to random numbers assigned to the ware. This procedure was fol-

Table 4. Pb (ppm) released from ceramic ware by 2 or 6% acetic acid

Av. leach temp., °C[a]	Makeup vol., mL	Acetic acid concn, %	Contact time, h: 2[a]	24	48
99.5	25	2.0	0.75	0.78	0.65
102.3	60	2.0	0.59	0.48	0.58
99.0	21	6.0	0.86	0.94	0.93
103.5	44	6.0	0.98	1.08	1.04

[a] 2 h heating period.

lowed to reduce or eliminate the shipment of a run of consecutively manufactured samples to any single collaborator.

The following were to be reported by the collaborators:

(1) the quantities of Pb and Cd (mg/L) found in both the 2 and 24 h samples

(2) the delay in hours (approximate) between the removal of the aliquot from the bulk solution and the time of assay by AAS

(3) the volume of 4% acetic acid used in each ware

(4) the volume of 4% acetic acid required to reestablish the leach volume after heating; records and calculations were to be sent

(5) the manufacturer of the AAS instrument and the approximate time it had been in service

(6) the kind of light source used with the AAS instrument: hollow cathode lamp, electrodeless discharge lamp, etc.

(7) all worksheets and calculations were to be submitted.

In the collaborative study, leach solutions were all analyzed after the 2 h heating period and again after 24 h total contact time. Analysis after 2 h, as outlined below, was adopted interim official first action.

Cadmium and Lead in Cookware
Hot Leach Atomic Absorption Method
Interim Official First Action
WHO-AOAC Method

25.D11 *Principle*

Enameled and ceramic cookware contg 4% acetic acid is heated by elec. hot plate (or by internal heating elements, if present, that are not exposed to the leach solution) to produce slow boil or simmering of solv. soln for 2 h. Pb and Cd in extg solv. are detd by AAS.

25.D12 *Apparatus*

See **25.031** plus the following:

(a) *Hot plate.*—Thermoline Model HP-A1915B (Thermoline Corp., Dubuque, IA 52001), or equiv.

(b) *Variable transformer.*—Cat. No. 09-521-100 (Fisher Scientific Co.), or equiv.

25.D13 *Reagents*

See **25.032.** Use only deionized distd H_2O.

Plus (e) *Acetic acid.*—Glacial.

25.D14 *Cleaning of Laboratory Glassware*

After normal cleaning, soak all glass and plastic ware used to prep., transfer, or store anal. solns in HNO_3–H_2O (4 + 6) ≥ 24 h; thoroly rinse with H_2O before use.

25.D15 *Preparation of Standard*

See **25.033.**

Samples of ware must be free of grease or other material which could influence test. Gently wash sample with detergent soln, using pad of absorbent cotton. Rinse ware thoroly with H_2O and let drain dry.

Fill volume of ware is $^2/_3$ of vol. required to fill ware to overflowing or to cover rest, if one is present.

25.D16 *Extraction*

Fill ware to $^2/_3$ total vol. with H_2O; cover with self-cover or clean sheet of opaque borosilicate glass to prevent evapn of soln. When leach soln is to be analyzed for Cd, ensure that light is excluded from test surface. Heat on hot plate adjusted by variable transformer to produce simmer or slow boil of leaching soln, or use internal heating element, if present. Also use variable transformer to prevent excessively rapid boiling in ware containing heating elements. If contained heating element is not able to produce temp. high enough to boil soln, then highest temp. reached is test temp.

When boiling or highest temp. has been reached, add sufficient glacial acetic acid to make soln 4% acetic acid, cover, and continue heating 2 h.

At end of 2 h, re-establish initial vol. of solv. with 4% acetic acid. Dip-stick (glass rod marked for depth of soln required) is useful for replacing losses. Stir thoroly and remove test sample at once.

25.D17 *Determination*

See **25.034.**

Comments of Collaborators

Several collaborators submitted comments on the hot leach method. One expressed concern that vapors from a large number of boiling acetic acid solutions might be a hazard to personnel and could corrode laboratory instruments. The user should make provisions to eliminate this hazard.

Other collaborators commented on the ex-

Table 5. Collaborative results for Pb and Cd (ppm) in enameled ware with lids

Lab.	Total ware vol., mL	Fill vol., mL: Total	Fill vol., mL: Acetic acid	Delay in assay, h	Assay time, h	Pb[a] 1	Pb 2	Pb 3	Pb 4	Pb 5	Pb 6	Cd 1	Cd 2	Cd 3	Cd 4	Cd 5	Cd 6
1	1740	1209	49.0	1	2	1.69	2.57	2.89	2.76	2.03	2.68	0.35	0.55	0.64	0.61	0.42	0.59
				0.5	24	1.69	2.53	2.86	2.76	1.98	2.68	0.35	0.55	0.63	0.60	0.42	0.59
						(37)	(34)	(15)	(47)	(5)	(16)						
2	1800	1200	—[b]	22.5	2	2.65	2.97	2.65	3.08	2.78	2.86	0.60	0.73	0.60	0.67	0.63	0.66
				22.5	24	2.65	2.89	2.67	3.03	2.85	2.96	0.60	0.66	0.61	0.74	0.64	0.65
						(250)	(250)	(250)	(250)	(250)	(250)						
3	1000	1041.6	41.6	28	2	3.6	4.3	3.7	2.7	3.6	4.4	0.88	1.14	1.03	0.76	0.99	1.24
				28	24	3.4	4.5	4.1	2.9	3.6	4.4	0.86	1.18	0.99	0.69	0.98	1.21
						(0)	(0)	(0)	(0)	(0)	(0)						
4	—[b]	1042	[b]	22.5	2	3.28	3.45	3.30	3.18	3.78	4.10	0.71	0.70	0.72	0.71	0.86	0.84
				24.5	24	3.28	3.45	3.60	3.18	3.85	4.10	0.71	0.70	0.72	0.72	0.86	0.84
						(500)	(500)	(500)	(500)	(500)	(500)						
5	1813[c]	1260	51.0[c]	—[b]	2	3.1	3.1	3.3	3.0	2.5	3.0	0.76	0.74	0.76	0.76	0.58	0.70
				—[b]	24	3.2	3.2	3.3	3.0	2.6	3.0	0.76	0.75	0.75	0.75	0.58	0.70
						(103)	(105)	(108)	(105)	(106)	(105)						
6	—[b]	1319	—[b]	1.5	2	3.01	2.20	2.65	3.37	2.29	2.92	0.66	0.47	0.61	0.77	0.53	0.65
				1.5	24	2.74	1.91	2.51	3.10	2.03	2.86	0.65	0.45	0.59	0.76	0.52	0.62
						(89)	(60)	(70)	(130)	(30)	(230)						
7	—[b]	1229[c]	49.7[c]	0.3	2	2.6	2.1	2.3	2.4	3.0	2.6	0.61	0.40	0.43	0.54	0.67	0.55
				0.3	24	2.6	2.1	2.3	2.4	3.0	2.6	0.61	0.40	0.43	0.54	0.67	0.55
						(100)	(77)	(160)	(141)	(95)	(66)						
8	—[b]	1190	—[b]	22	2	3.68	3.08	3.39	3.44	3.18	3.27	0.77	0.65	0.71	0.73	0.64	0.69
				22	24	3.72	3.13	3.44	3.44	3.32	3.41	0.77	0.65	0.71	0.73	0.67	0.70
						—[b]	—[b]	—[b]	—[b]	—[b]	—[b]						
9	1700	1135	47.7	[b]	2	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]
				[b]	24	2.5	2.45	2.4	2.9	2.75	2.9	0.48	0.47	0.47	0.55	0.48	0.57
						—[b]	—[b]	—[b]	—[b]	—[b]	—[b]						
10	[b]	1167	—[b]	70	2	1.66	2.28	1.33	1.26	1.48	1.44	0.44	0.48	0.28	0.26	0.31	0.31
				70	24	1.67	2.26	1.33	1.26	1.55	1.52	0.35	0.47	0.28	0.26	0.32	0.31
						(0)	(0)	(0)	(0)	(0)	(0)						
11	1800	1250	50.0	23	2	2.33	2.38	2.51	2.38	2.50	2.44	0.49	0.49	0.53	0.49	0.54	0.51
				1	24	2.37	2.43	2.55	2.44	2.60	2.51	0.50	0.50	0.54	0.50	0.55	0.52
						(200)	(150)	(200)	(150)	(100)	(100)						
12	1800	1250	50.0	0.75	2	2.33	2.32	2.26	1.63	2.25	2.17	0.50	0.51	0.46	0.33	0.49	0.48
				2	24	2.65	2.64	2.52	1.80	2.54	2.46	0.54	0.55	0.47	0.35	0.50	0.49
						(113)	(130)	(136)	(118)	(124)	(132)						
13	—[b]	1269	—[b]	1	2	4.68	4.42	4.35	3.85	4.15	4.23	1.12	1.03	0.96	0.88	1.06	0.92
				1	24	4.63	4.55	4.39	3.91	4.09	4.30	1.19	1.00	0.95	0.84	1.03	0.90
						(145)	(137)	(162)	(168)	(135)	(124)						
14	—[b]	1000	—[b]	22.5	2	3.75	3.50	3.75	3.67	3.67	3.58	0.81	0.74	0.85	0.79	0.81	0.76
				0.25	24	3.75	3.50	3.67	3.58	3.58	3.58	0.79	0.73	0.82	0.76	0.81	0.73
						(185)	(95)	(180)	(165)	(200)	(140)						

[a] Makup volume, mL, in parentheses.
[b] Not reported.
[c] Average.

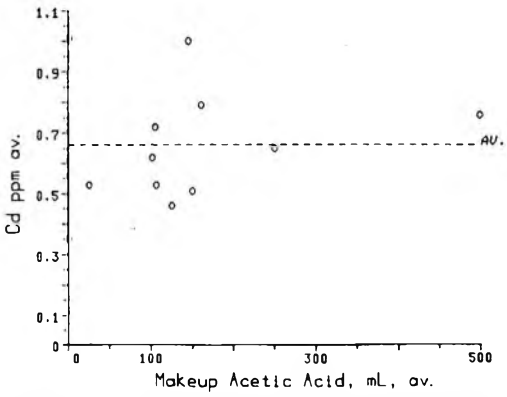

Figure 4. Scatter plot of average Cd found by collaborators vs quantity of acetic acid solution required to restore initial leach volume in enameled ware.

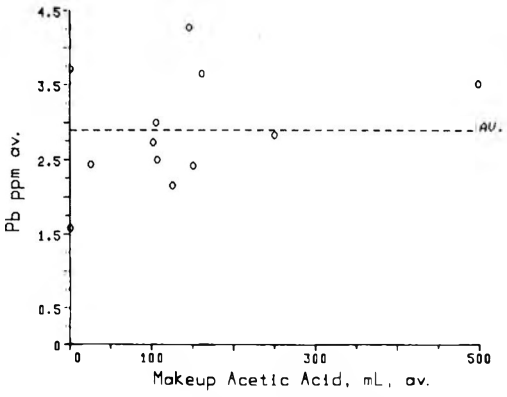

Figure 5. Scatter plot of average Pb found by collaborators vs quantity of acetic acid solution required to restore initial leach volume in enameled ware.

perimental difficulties of accurately measuring the hot acetic acid solution required to restore the volume of leach solution during and after the heating period. Several workers used the dipstick procedure described above for this purpose.

Results and Discussion

The results of the collaborative study of the leaching of the enameled ware are given in Tables 5 and 6 and for the ceramic ware in Tables 7 and 8.

The analysis of variance for the enameled ware in Table 6 shows that there were no significant differences between the means or the variances when the 2 h results were compared with the 24 h results.

The analysis of variance for the assay of the ceramic cookware is given in Table 8. It also shows no significant differences between the means or the variances when the 2 h results were compared with the 24 h results.

An evaluation of the volume of 4% acetic acid required to restore the leach volume showed that there was no significant relationship between the average metal content and the makeup volume added. This lack of relationship is shown by scatter plots. Figure 4 is a plot of the relationship of the average Cd found by each collaborator in 24 h samples in the enameled ware against the average quantity of acetic acid solution required to restore the volume after the heating period. Figure 5 is a similar scatter plot for Pb released from the enameled ware and Figure 6 for the Pb released from the ceramic ware. None of the examples showed a trend of

Table 6. Enameled ware: summary of analysis of variance

	Pb		Cd	
Parameter[a]	2 h	24 h	2 h	24 h
n	78	84	78	84
$\bar{x}$ (ppm)	2.94	2.94	0.6620	0.6471
lab. s^2	0.5470	0.4993	0.0371	0.0340
*lab. s	0.7396	0.7066	0.1926	0.1843
% CV	25.2	24.0	29.1	28.5
error s^2	0.1129	0.1107	0.0080	0.0083
**error s	0.3360	0.3327	0.0892	0.0913
% CV	11.4	11.3	13.5	14.1
overall s^2	0.6126	0.5799	0.0426	0.0402
***overall s	0.8123	0.7810	0.2124	0.2057
% CV	27.6	26.6	32.1	31.8

[a] Symbols used: $\bar{x}$ = overall mean; lab. s^2 = between-laboratory variance; error s^2 = sample variance; s = standard deviation; overall s^2 = variance from analysis of one sample by one laboratory; CV = coefficient of variation; * = between-laboratory standard deviation; ** = repeatability, within-laboratory standard deviation; *** = reproducibility, overall standard deviation.

Table 7. Collaborative results for Pb and Cd (ppm) in ceramic cookware with covers

Lab.	Total ware vol., mL	Fill vol., mL: Total	Fill vol., mL: Acetic acid	Delay in assay, h	Assay time, h	Pb[a] 1	Pb 2	Pb 3	Pb 4	Pb 5	Pb 6	Cd 1	Cd 2	Cd 3	Cd 4	Cd 5	Cd 6
1	250	167	7.0	4	2	0.84	0.89	0.93	0.91	0.82	0.83	0.01	0.01	0.01	0.00	0.00	0.00
				0.5	24	0.73	0.79	0.79	0.89	0.66	0.69	0.01	0.01	0.00	0.00	0.00	0.00
						(73)	(92)	(79)	(94)	(105)	(89)						
2	350	233	—[b]	22.5	2	0.87	0.95	1.20	0.86	0.82	1.04	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
				22.5	24	0.87	1.03	1.28	0.90	0.83	1.12	0.01	0.01	0.01	0.01	0.01	0.01
						(55)	(55)	(55)	(55)	(55)	(55)						
3	170	177.1	7.1	28	2	2.3	0.8	1.1	1.2	1.1	0.7	0	0	0	0	0	0
				28	24	0.9	0.7	0.8	0.9	1.2	0.7	0	0	0	0	0	0
						(20–50)											
4	—[b]	146	—[b]	22.5	2	0.0	0.0	0.0	0.0	0.0	0.0	0	0	0	0	0	0
				24.5	24	0.0	0.0	0.0	0.0	0.0	0.0	0	0	0	0	0	0
						(146)	(146)	(146)	(146)	(146)	(146)						
5	251[d]	174[d]	7.0[d]	—[a]	2	1.4	0.9	0.8	1.1	1.3	0.8	0	0	0	0	0	0
				—[a]	24	1.3	0.8	0.8	1.1	1.4	1.0	0	0	0	0	0	0
						(164)	(175)	(176)	(175)	(166)	(170)						
6	—[b]	222	—[b]	1.5	2	0.57	0.84	0.48	0.66	0.66	0.66	0	0	0	0	0	0
				1.5	24	0.60	0.84	0.60	0.48	0.72	0.84	0	0	0	0	0	0
						(52)	(74)	(56)	(46)	(48)	(42)						
7	—[b]	179[d]	7.0	0.3	2	1.0	1.0	1.2	0.8	1.2	0.8	0	0	0	0	0	0
				0.3	24	1.3	1.0	1.3	0.8	1.3	0.8	0	0	0	0	0	0
						(106)	(98)	(80)	(45)	(45)	(64)						
8	—[b]	177	—[b]	22	2	0.8	0.78	0.67	0.94	—[b]	—[b]	—[f]	—[f]	—[f]	—[f]	—[b]	—[b]
				22	24	0.8	0.83	0.78	0.99	—[b]	—[b]	—[f]	—[f]	—[f]	—[f]	—[b]	—[b]
						—[b]	—[b]	—[b]	—[b]	—[b]	—[b]						
9	250	167	7.0	0.5	2	0.55	0.50	0.55	0.50	0.60	—[e]	—[c]	—[c]	—[c]	—[c]	—[c]	—[e]
				—[b]	24	0.55	0.50	0.55	0.50	0.60	—[e]	—[c]	—[c]	—[c]	—[c]	—[c]	—e
						(59)	(28)	(86)	(74)	(59)							
10	—[b]	184.4	—[b]	72	2	0.61	0.85	0.61	0.76	0.81	0.63	0	0	0.01	0.01	0.02	0.01
				72	24	0.62	0.85	0.71	0.82	0.75	0.63	0	0	0.01	0	0	0
						(0)	(0)	(0)	(0)	(0)	(0)						
11	240	166.7	6.7	23	2	0.48	0.76	0.57	0.80	0.79	—[b]	—[c]	—[c]	—[c]	—[c]	—[c]	—[b]
				1	24	0.50	0.77	0.59	0.84	0.82	—[b]	—[c]	—[c]	—[c]	—[c]	—[c]	—[b]
						(30)	(25)	(20)	(30)	(35)	—[b]						
12	270	187.6	7.6	ca. 1	2	1.71	1.31	1.93	1.78	1.32	0.96	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]
				2	24	1.83	1.54	2.16	1.94	1.38	1.07	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]
						(43)	(35)	(51)	(33)	(39)	(46)						
13	—[b]	177	—[b]	2	2	1.17	1.46	1.04	1.08	1.34	1.09	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]
				2	24	1.29	1.48	1.11	1.19	1.38	1.16	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]
						(32)	(38)	(29)	(32)	(34)	(29)						
14	—[b]	167	—[b]	0.25	2	0.83	0.91	1.14	1.21	0.87	0.91	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
				2.5	24	0.80	0.92	1.09	1.15	0.81	0.88	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
						(90)	(90)	(70)	(110)	(110)	(80)						

[a] Makeup volume, mL, in parentheses.
[b] Not reported.
[c] <0.01 ppm.
[d] Average.
[e] Broken.
[f] <0.03 ppm.
[g] <0.02 ppm.

Table 8. Ceramic cookware: summary of analysis of variance

Parameter[a]	Pb[b] 2 h	Pb[b] 24 h
n	80	80
$\bar{x}$ (ppm)	0.9992	1.0026
lab. s^2	0.2720	0.2881
*lab. s	0.5216	0.5367
% CV	52.2	53.5
error s^2	0.0590	0.0420
**error s	0.2429	0.2050
% CV	24.3	20.4
overall s^2	0.3147	0.3128
***overall s	0.5753	0.5745
% CV	57.6	57.3

[a] Symbols used: $\bar{x}$ = overall mean; lab. s^2 = between-laboratory variance; error s^2 = sample variance; s = standard deviation; overall s^2 = variance from analysis of one sample by one laboratory; CV = coefficient of variation; * = between-laboratory standard deviation; ** = repeatability, within-laboratory standard deviation; *** = reproducibility, overall standard deviation.

[b] Cd levels below quantitation limit of method.

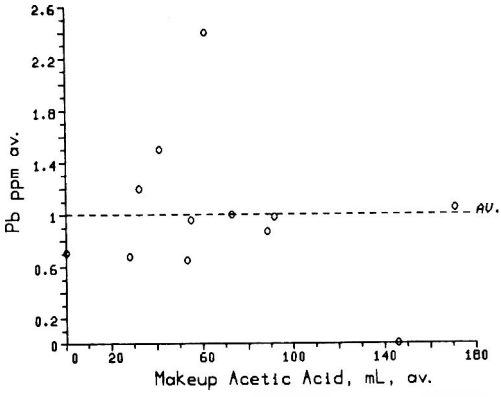

Figure 6. Scatter plot of average Pb found by collaborators vs quantity of acetic acid solution required to restore initial volume in ceramic ware.

metal release that correlated with the volume of the makeup acetic acid required.

Because an increasing quantity of metals was extracted with increasing temperature, we were concerned that the results from the Food and Drug Administration (FDA) laboratory in Denver (Laboratory 1) would vary significantly from the results obtained in laboratories at lower altitudes. This was not the case, and the results from Denver did not differ significantly from those from other laboratories. Four collaborative laboratories (at lower altitudes than Denver) reported less average Pb from ceramic ware and less average Cd from the enameled ware. Insufficient Cd was leached from ceramic ware to draw a conclusion concerning it.

Within the context of the limitations imposed by the special ware used, the results of these tests show that essentially all of the Cd and Pb is released during the 2 h of active heating and that little is released during the 22 h RT contact period. Elimination of the 22 h RT contact period results in a significant saving of the time and space needed to carry out the test procedure.

The 2 h heating period was arbitrarily chosen to approximate the typical cooking time for the type of ware tested. From a toxicological standpoint, other hot leach periods could be used so that the results obtained would provide a better measure of the risks involved in the actual use of the ware being examined.

This collaborative study was conducted using specially formulated glazes and enamels prepared to release sufficient Pb and/or Cd for direct AAS measurement. The universal applicability of this hot leach method is yet to be tested on cookware prepared with commercially available glazes and enamels. Glazes, if properly prepared, should normally release only small amounts of Pb and Cd when analyzed by the AOAC-RT leach method now in general use. Enamels that are manufactured in the United States and intended for use on food contact surfaces normally do not contain Pb and Cd.

Recommendations

It is recommended that the hot leach method, using the 2 h leach period, be adopted interim official first action.

It is also recommended that the method be submitted to the American Society for Testing and Materials, the American National Standards Institute, and the International Standards Organization for consideration and possible approval.

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Determination of Copper, Nickel, and Chromium in Foods

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A collaboratively studied method for Pb, Cd, As, Se, and Zn that uses a closed system digestion technique has now been extended to include 3 additional elements, Cu, Ni, and Cr. Cu is determined by either atomic absorption spectrophotometry or anodic stripping voltammetry, depending on the concentration. Ni and Cr are determined by differential pulse polarography. Analysis of National Bureau of Standards reference materials by this procedure gives values in close agreement with the accepted values. Recoveries from applesauce and chicken spiked at 0.6-4 μg/g are in the 92-101% range. The sensitivity of the multielement procedure is 0.34, 0.14, and 0.24 μg/g for Cu, Ni, Cr, respectively, at the 90% confidence level.

We previously (1) developed a multielement analytical method for the determination of lead, cadmium, arsenic, selenium, and zinc in foods; a collaborative study of this method resulted in the method being adopted as official first action. Briefly, the method involves sample digestion in a closed system, and, after some additional treatment, determination of the elements by one of the commonly available techiques. Cadmium and lead are determined by anodic stripping voltammetry (ASV), zinc by conventional atomic absorption spectrophotometry (AAS), and arsenic and selenium by hydride AAS. We now report methods which extend this procedure to include 3 more metals: copper, nickel, and chromium. Copper is determined by ASV; nickel and chromium are determined by differential pulse polarography (DPP). These metals are easily determined by AAS, but because of the low concentrations at which they are usually found in foods, conventional AAS may not be sufficiently sensitive.

METHOD

Apparatus

As described previously (1) with the following differential pulse polarographic conditions: Ni, drop time 0.5 s; modulation amplitude 50 mv; scan rate 2 mv/s. Cr, drop time 1 s; modulation amplitude 50 mv; scan rate 5 mv/s. Microsampling tube for the determination of Cu: Break off a 2.5 cm length of tube from narrow end of Pasteur pipet, fire-polish top end of tube, and seal bottom of tube with flame.

Reagents

All reagents are reagent grade unless otherwise indicated; water is double-distilled in an all-glass still.

(a) *Standard copper solutions.*—(*1*) 1 mg Cu/L. Dissolve 1.000 g copper powder (m3N, Ventron Corp., Alfa Products, Beverly, MA 01915) in 20 mL HNO_3 (1 + 1) in 1 L volumetric flask and dilute to volume with water. (*2*) *Working solutions.*—5 μg and 20 μg Cu/L. Dilute aliquot of solution (*1*) with water. Use the same day. Other concentrations of working solutions may be necessary.

(b) *Standard nickel solutions.*—(*1*) 1 mg Ni/L. Dissolve 1.000 g nickel powder (t3N, Ventron Corp.) in 20 mL HNO_3 (1 + 1) in 1 L volumetric flask and dilute to volume with water. (*2*) *Working solutions.*—5 μg Ni/L. Dilute aliquot of solution (*1*) with water. Use the same day.

(c) *Standard chromium solution.*—(*1*) 1 mg Cr (VI)/L. Dissolve 2.828 g $K_2Cr_2O_7$ (Fisher Scientific Co., Fairlawn, NJ) in water in 1 L volumetric flask. Add 1 NaOH pellet and dilute to volume. (*2*) 10 μg Cr (VI)/L. Dilute aliquot of solution (*1*) with water. Use the same day.

(d) *Dimethyl glyoxime (DMG) solution.*—1%. Dissolve 0.1 g DMG (Fisher Scientific Co.) in 10 mL ethanol.

(e) *Ammonium citrate buffers.*—(*1*) 1M, pH 10.7. Dissolve 22.6 g ammonium citrate (Fisher Scientific Co.) in 100 mL water. Adjust to pH 10.7 with NH_4OH. (*2*) 0.1*M*, pH 9. Dissolve 2.26 g ammonium citrate in 100 mL water. Adjust to pH 9 with NH_4OH.

(f) *Lithium hydroxide.*—$LiOH \cdot H_2O$ (Fisher Scientific Co.).

(g) *Ion exchange resin.*—Chelex-100, sodium form, 100-200 mesh (Bio-Rad Laboratories, Richmond, CA). Pour aqueous slurry of resin into a plastic 0.8 cm id column to height of 3 cm. Pass 0.1M ammonium citrate buffer (pH 9) through column until pH of effluent is that of buffer.

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W. Holak is the Associate Referee on Multielement Determination after Closed System Digestion.

Closed System Sample Digestion

Digest 0.5 g sample (dry weight basis) as described previously (1).

Determination of Copper by AAS

Add 0.1 mL (Eppendorf pipet or equivalent) of closed system-digested sample and 0.02 mL water to microsampling tube. Operate atomic absorption spectrophotometer according to manufacturer's specifications, using air–acetylene flame and recorder. Aspirate solution in microsampling tube and record absorbance, using scale expansion if necessary. Aspirate 0.1 mL sample and 0.02 mL water 2 additional times to obtain triplicate readings. Similarly obtain triplicate readings for 0.1 mL sample and 0.02 mL copper working standard. Concentration of standard should be adjusted to at least double sample signal while still working in linear range. Run reagent blank in same manner as sample. μg Cu in microsampling tube = $[A_1/(A_2 - A_1)] \times B$ where A_1 = average absorbance of sample; A_2 = average absorbance of sample spiked with standard; B = μg Cu added to microsampling tube. Similarly, calculate μg Cu in microsampling tube contributed by the blank and subtract from sample; then calculate μg Cu/g.

Determination of Copper by ASV

Determine Cu as described for Pb and Cd (1), but instead of dissolving residue in 5.0 mL HNO_3 (0.5 mL/L), use 5.0 mL 0.1M HCl and transfer to polarographic cell with 5 ml 0.1M HCl. Copper peak appears at ca −0.05V vs Ag–AgCl.

Determination of Nickel by DPP

To solution used to determine Cu, add 1 mL 1M ammonium citrate buffer (pH 10.7) and 0.05 mL DMG solution (1%), and bubble nitrogen through solution for 2 min. Scan voltage from −0.8 to −1.1V according to conditions given for Ni under *Apparatus*. Nickel peak appears at about −0.97 V vs SCE. Quantitate by method of standard additions as described for ASV (1). Do not exceed linear range of about 0.1 μg Ni/mL.

Determination of Chromium by DPP

Treat aliquot of closed system-digested sample with $KNO_3/NaNO_3$ as described previously (1) but do not dissolve solidified melt in acid. Instead, proceed as in (**a**) (Cr > 1 ppm) or (**b**) (Cr < 1 ppm) below:

(**a**) Pipet 10 mL 0.1M ammonium citrate buffer (pH 9), cover with watch glass, and swirl to dissolve residue. Filter, if necessary. Pipet 7 mL solution or filtrate into polarographic cell and bubble nitrogen through solution for 5 min; then add 0.14 g $LiOH{\cdot}H_2O$ through hole in cell top, continuing to bubble nitrogen until $LiOH{\cdot}H_2O$ dissolves. pH of solution should be ≥13. Scan voltage from −0.5 to −1.0 V according to conditions given for Cr in *Apparatus*. Cr peak appears at ca −0.80 V vs SCE. Quantitate by method of standard additions as described for ASV (1).

(**b**) Dissolve residue in 5 mL 0.1M ammonium citrate buffer (pH 9), pass through Chelex-100 column, and collect effluent in polarographic cell. Wash beaker with 5 mL buffer and pass through column as before. Bubble buffer-washed nitrogen through solution for 10 min and scan voltage from −0.15 to −0.45 V according to conditions given for Cr under *Apparatus*. Cr peak appears at ca −0.28 V vs SCE. Quantitate by method of standard additions as decribed for ASV (1).

Table 1. Analysis [a] of Standard Reference Materials (SRMs), μg/g

	Found	NBS value [b]
SRM 1570, Spinach		
Cu	10.8	12 ± 2
Ni	6.4	(6)
Cr	3.9	4.6 ± 0.3
SRM 1577, Bovine Liver		
Cu	196	193 ± 10
SRM 1567, Wheat Flour		
Cu	1.8	2.0 ± 0.3
Ni	0.23	(0.18)
SRM 1571, Orchard Leaves		
Cu	12.0	12 ± 1
Ni	1.8	1.3 ± 0.2
Cr	1.9	(2.3)

[a] Single determinations.
[b] Values in parentheses are uncertified.

Results and Discussion

The work described shows that the general method used for the analysis of foods for Pb, Zn, Cd, Se, and As can be extended to include determination of Cu, Ni, and Cr. Analysis of National Bureau of Standards reference materials by this procedure gave values in close agreement with the accepted values (Table 1). Recoveries from applesauce and chicken (Table 2) were in the range of 92–101%. Figures 1–3 illustrate ASV scans of copper and differential pulse polaro-

Table 2. Analysis [a] of strained applesauce and strained chicken samples and spikes, μg/g

	Found in unspiked sample	Added	Total found	Rec., %
		Applesauce		
Cu	0.03 ± 0.07	2.00	2.05 ± 0.15	101
Ni	2.09 ± 0.16	2.50	4.56 ± 0.32	99
Cr[b]	0.01 ± 0.01	0.60	0.56 ± 0.067	92
		Chicken		
Cu	0.62 ± 0.11	4.00	4.41 ± 0.13	94
Ni	0.15 ± 0.05	0.60	0.72 ± 0.03	95
Cr[c]	0.03 ± 0.04	2.00	2.03 ± 0.09	100

[a] In quadruplicate.
[b] Ammonium citrate, pH 9, supporting electrolyte.
[c] LiOH supporting electrolyte.

graphic scans of nickel and chromium, respectively, as derived from Reference Material 1570 (Spinach). Figure 4 shows Cr DPP scans in ammonium citrate buffer, pH 9; Figure 5 shows Cu atomic absorption microsampling signals.

Sample size was increased from the previously (1) used 0.3–0.5 g (dry basis) to improve sensitivity and because more sample was needed to determine 3 additional elements. Sample size limitation is given on the dry basis to ensure that the decomposition vessel is not overloaded with organic material. However, since the water content of food varies, the sample weight taken may be 0.5–5.0 g on wet or "as is" basis.

The sensitivity of the ASV/DPP method was estimated (2) by using the formula, $t = \Delta s/\delta\sqrt{2}$, where t = value obtained from table of t-values with $N = 1$ degree of freedom, Δs = difference between sample and blank determinations, and δ = standard deviation of the blank. Average blanks in sample cells in this study were 0.083 ± 0.019 μg Cu, 0.059 ± 0.008 μg Ni, and 0.065 ± 0.013 μg Cr in ammonium citrate supporting electrolyte. In LiOH supporting electrolyte, Cr background variation corresponded to ±0.014 μg Cr. Solving for Δs (t = 6.413), 0.16 μg Cu, 0.07 Ni, and 0.13 or 0.12 μg Cr can be detected with 90% certainty. Assuming that 2.5 g sample (as-is basis) is taken for the closed system digestion and 2 mL aliquots are taken for the determinations, the sensitivity of the method is 0.32 μg Cu/g, 0.14 μg, Ni/g, 0.37 μg Cr/g in LiOH supporting electrolyte and 0.24 μg/g in ammonium citrate supporting electrolyte at the 90% confidence

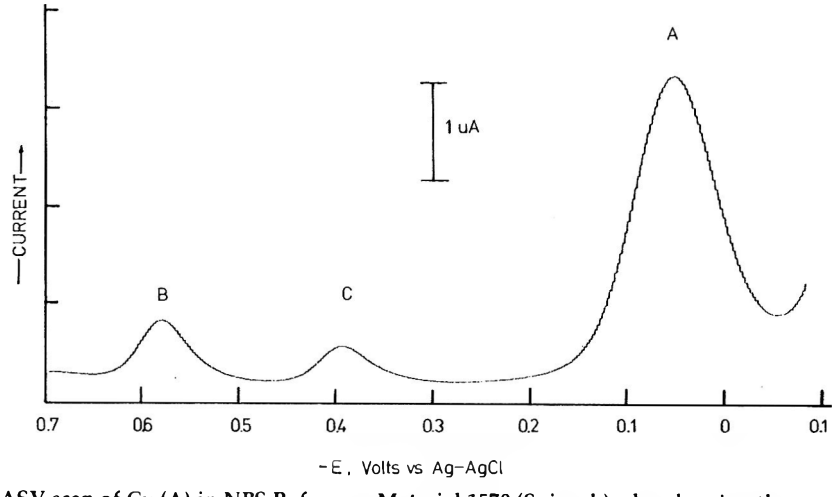

Figure 1. ASV scan of Cu (A) in NBS Reference Material 1570 (Spinach), also showing the presence of Cd (B) and Pb (C).

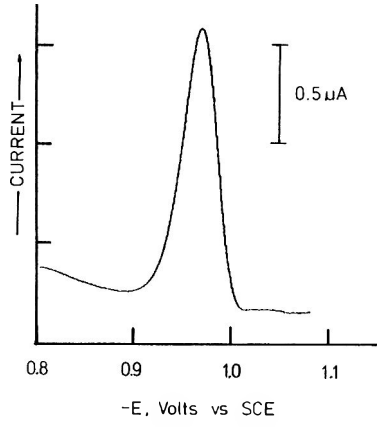

Figure 2. DPP scan of Ni in same sample as in Figure 1.

level. With this scheme, all 8 elements (Pb, Cd, As, Se, Zn, Cu, Ni, and Cr) can be determined from a single digest. If an element needs to be determined at a lower level, that determination can be optimized by taking a larger aliquot.

If the concentration of copper is approximately 1 μg/mL, it can easily be determined by AAS after the closed system sample digestion. The microsampling technique (3) is used to reduce the consumption of the sample.

A fast instrument response should be used to record transient copper signals. At concentrations suitable for AAS, there is no contamination from the micropipets. At lower concentrations, Cu can be determined by ASV in approximately 0.1N HCl. In this supporting electrolyte, the Cu peak appears at a more negative potential than

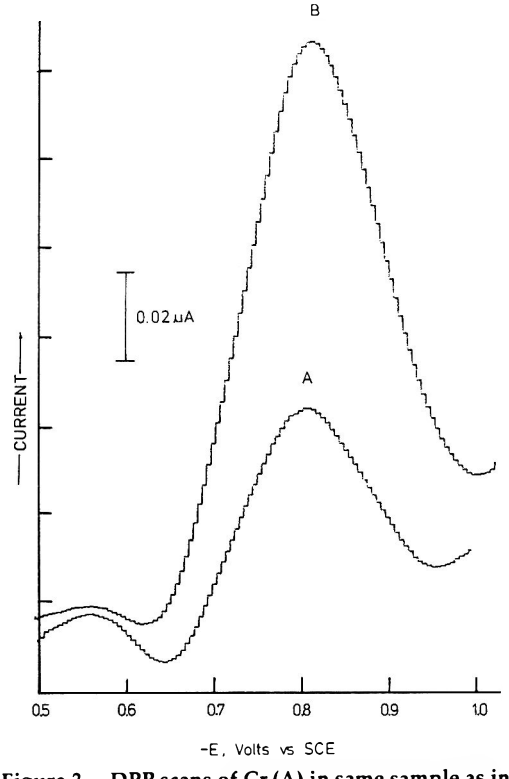

Figure 3. DPP scans of Cr (A) in same sample as in Figure 1. Peak B is from standard addition of 0.8 μg Cr to cell. LiOH supporting electrolyte.

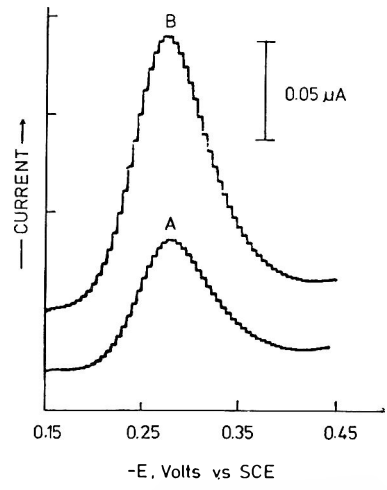

Figure 4. DPP scans of Cr in applesauce spike (A). Peak B is from standard addition of 0.5 μg Cr to cell. Ammonium citrate, pH 9, supporting electrolyte. electrolyte.

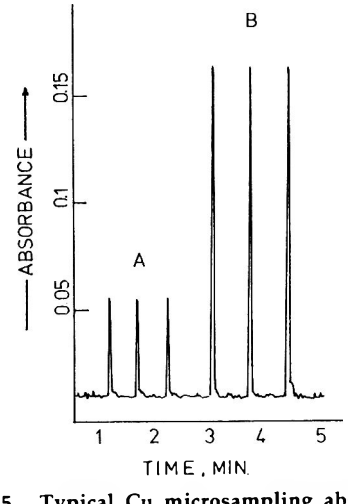

Figure 5. Typical Cu microsampling absorbance signals in sample (A) and after standard additions (B) of 5 μg/mL Cu standards. (See text.)

in a non-complexing medium such as dilute HNO_3, as it is removed from the interference caused by the oxidation of mercury. Since 0.1N HCl is also suitable for the determination of Pb and Cd, all 3 elements can be determined at the same time.

DPP determination of nickel in foods is made possible by the fact that addition of DMG to the solution (pH $\geq$ 9.3) results in great enhancement of the nickel signal (4). Other metals commonly found in foods do not interfere with the nickel determination. The linear range, however, is quite narrow (0 to ~0.1 μg/mL) and should not be exceeded. By performing the quantitation by the method of standard additions (1), linearity is easily ascertained. If linearity is exceeded, a smaller aliquot can be taken for the determination or the solution may be diluted with more buffer solution.

The most commonly used supporting electrolyte (5) for chromium (VI) is 0.1-1M NaOH (pH 13-14). Reagent grade LiOH instead of reagent grade NaOH was used in this procedure because the former could be used without additional purification. This chemical is added to the solution after the removal of oxygen to minimize H_2O_2 formation which may arise in the presence of Hg in strongly alkaline solution (5). Solid LiOH rather than LiOH solution is added so that oxygen is not introduced; H_2O_2 gives an interfering peak at about -0.9 V. It is essential that the nitrogen used for deaeration be oxygen-free. We have also eliminated the peak at -0.9 V by bubbling helium through the solution. Although Pb gives a peak at about -0.7 V in an alkali hydroxide supporting electrolyte, it would have to be present at a much higher concentration than Cr to present an interference problem. After the sample is digested with $KNO_3/NaNO_3$, the residue should not be dissolved in an acid solution. Chromium present at this point is in the required hexavalent state, but if the solution is acidified, the nitrous acid formed (from $KNO_2/NaNO_2$) will reduce Cr (VI) to Cr (III). This can be demonstrated by observing the color change (from orange to violet) when $K_2Cr_2O_7$ is heated in an acid solution of $NaNO_2$.

At the sub-ppm level, ammonium citrate (pH 9) supporting electrolyte may be preferable. In this medium, however, copper interferes, but it can be conveniently removed from solution by the described procedure with chelating ion-exchange resin. This resin strongly adsorbs transition elements but $Cr_2O_7^=$ is not adsorbed.

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EXTRANEOUS MATERIALS

Automated Analysis of Flour Extracts for Uric Acid and Its Correlation With Degree of Insect Defilement

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An automated method is described for quantitating uric acid in a sodium acetate extract of flour. The difference in color intensity developed in a flour extract treated with the enzyme uricase and one that is not treated is measured spectrophotometrically. A mean recovery of 97.1% was obtained for 50 g flour samples spiked with 1 mg uric acid. The method can measure as little as 50 μg uric acid in 50 g flour and is used to establish a correlation between the uric acid in the flour and the number of insect fragments and excreta pellets present. A 42-data point least squares straight line having a slope of 1.62 and a correlation coefficient of 0.750 (99.9-100% valid) is used to establish the relationship of uric acid to insect defilement.

Accurate measurement of the degree to which flour has been insect-defiled can be tedious and time consuming. The official AOAC methods can be classified by their final determinative step as either microscopic (1–3) or chemical (4). The microscopic methods, which involve counting of insect fragments (1, 2) or insect excreta pellets (3), are wearisome and require special entomological training. Moreover, the relationship of insect fragment count to the amount of insect defilement in a food is not clear. For example, a large number of fragments present may not necessarily mean a large number of insects contaminating the food, but rather a small number of insects broken into many fragments. A truer correlation exists between the number of insect excreta pellets present and the degree of insect defilement. A large number of pellets indicates conversion of a large amount of food material to insect excrement. It makes no difference whether the food was defiled by a long stay of a small number of insects or by a large number of insects during a short stay. In either case, the food is defiled to the same degree.

The uric acid content of flour provides an indication of the number of excreta pellets present and the number of insects broken up in the making of the flour (5–7). Determination of uric acid has been proposed (5–11) as a chemical index of insect infestation in a wide variety of foods.

The official AOAC chemical method for measuring uric acid in flour (4) is lengthy, involving time-consuming (16 h) digestion with hydrochloric acid–glutathione, extraction of uric acid, and measurement of absorption at 292 nm. Moreover, this method is applicable only to amounts of uric acid ≥4 mg/100 g sample. A study by Sen (5) indicated that adult and larval *Tribolium castaneum* (red flour beetle) excrete an average of 12.6 and 39.6 μg uric acid/day, respectively. It would take 10 insects (5 larvae and 5 adults) over 14 days to excrete 4 mg uric acid. Thus, the official method will detect only gross amounts of insect defilement and is inadequate for the majority of regulatory samples.

Both Laessig and coworkers (11) and Roy and Kvenberg (7) reported semiautomated analytical procedures that are suitable for routine determination of uric acid in a wide variety of *T. confusum* (confused flour beetle)-infested food products. The Laessig et al. procedure is sensitive to only 4 mg uric acid/100 g sample. Roy and Kvenberg reported the sensitivity of their method as "approximately 5 μg uric acid/g of sample . . ." (0.5 mg/100 g). However, the Roy and Kvenberg method specifies the analysis of only 1 g flour, which may not be a representative portion of the sample.

The uric acid procedure described here has been adapted from the Roy and Kvenberg method (7). The extraction has been modified so that a more representative 50 g flour sample is analyzed. The proposed method also has an improved sensitivity of 50 μg/50 g (0.1 mg/100 g) and corrects for the presence of potassium bromate, which is added to flour as a maturing agent (12). Adding sodium thiosulfate during sample preparation reduces the bromate (BrO_3^-), which interferes in the final color production step, to noninterfering bromide (Br^-).

The uric acid is extracted from the flour into 5% sodium acetate solution, and the flour extract is processed through a dual-channel automated

[1] Associate Referee on Insect Excreta in Flour.

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analyzer. Uric acid is determined colorimetrically.

METHODS

Apparatus

(a) *Automated equipment.*—Consisting of liquid sampler II, proportioning pump III, 2 manifolds 116-D638-01, SC colorimeter, 3 uricase coils T 10-0005-51, 120 V single pen recorder, and Cera Clear filters (Technicon Industrial Systems, Tarrytown, NY 10591).

(b) *Centrifuge.*—International type SB, size 1 (Damon/IEC Division, Needham Heights, MA 02194).

(c) *Wrist action shaker.*—Burrell Corp., Pittsburgh, PA 15219.

Reagents

(a) *Sodium thiosulfate solution.*—0.5 mg/mL.—Transfer 1.00 g $Na_2S_2O_3 \cdot 5H_2O$ to 2 L volumetric flask containing ca 1500 mL water. Dissolve in and dilute to volume with water.

(b) *Phosphotungstic acid solution.*—Transfer 50.0 g sodium tungstate to 1 L flask with ground-glass joint. Add 600 mL water and dissolve sodium tungstate. Place a few glass beads in flask. Add 80 mL 85% *o*-phosphoric acid and mix. Attach reflux condenser and boil gently 4 h. Cool to room temperature. Transfer contents to 1 L volumetric flask. Rinse reaction flask and transfer rinsings to volumetric flask. Dilute to volume with water. Filter through paper and store in 1 L brown bottle. (Commercially prepared solutions are available.)

(c) *Hydroxylamine hydrochloride.*—2%.—In 1 L volumetric flask dissolve 2.0 g hydroxylamine hydrochloride in ca 800 mL water. Dilute contents to volume with water. (Commercially prepared solutions are available.)

(d) *Sodium tungstate solution.*—25%.—In 1 L volumetric flask dissolve 250 g sodium tungstate in ca 800 mL water. Dilute to volume with water. (Commercially prepared 50% solutions can be diluted with water.)

(e) *Donor sodium borate solution.*—10%.—In 1 L volumetric flask containing 800 mL water dissolve 100 g $Na_2B_4O_7 \cdot 10H_2O$. Add water to bring volume to neck of flask. Add 1 mL Aerosol-22 (88% bioctyl sodium sulfosuccinate, Technicon) and slowly dilute to volume with water.

(f) *Recipient sodium borate solution.*—10%.—Prepare same as donor sodium borate solution but use 2 mL Aerosol-22.

(g) *Enzyme stabilizing reagent.*—Place 39.6 g ammonium sulfate crystals in 100 mL volumetric flask and add ca 70 mL water to dissolve crystals. Add 2.0 g ethylene glycol and dilute to volume with water.

(h) *Uric acid stock solution.*—0.1 mg/mL.—In 500 mL volumetric flask dissolve 50 mg uric acid in ca 400 mL 5% sodium acetate solution. If necessary, gently warm mixture in hot water bath to dissolve uric acid. Cool, dilute mixture to volume with sodium acetate solution, and filter. Store in brown bottle in refrigerator. Solution is stable 5 days.

(i) *Working standards.*—In separate volumetric flasks, dilute aliquots of stock solution with 5% sodium acetate solution to obtain concentrations of 8.0, 6.0, 4.0, 2.0, 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1 μg/mL. Refrigerated solutions can be used 3 days. Warm to room temperature before use.

Extraction

Weigh 50.0 g flour into 500 mL glass-stoppered flask and add 50 mL 1N HCl (slurry flour) and 50 mL sodium thiosulfate solution (slurry flour). Heat flask in 50–60°C water bath 30 min. Cool to room temperature and add 50 mL 1N NaOH (slurry flour) and 100 mL 5% sodium acetate solution. Stopper flask and gently shake 10 min on wrist-action mechanical shaker. Pour ca 40 mL into centrifuge tube and centrifuge at 1500 rpm 15 min. Filter supernate using Cera Clear and 16 × 160 mm culture tubes. Use filtered supernate for determination.

Determination

Solutions (3–4 mL) are sampled from 4 mL cups by liquid sampler II at rate of 30/h with sample-to-wash ratio of 2:1. A 40-cup carousel is used. Duplicate cups of the 10 working standard solutions are run, then 2 cups of reagent blank, and then duplicate cups of the samples. Sodium acetate solution is run between each carousel for 10 min to purge the system of contaminants.

Upon leaving the sampler, the flour extract stream is split in half (Figure 1). One half of the stream flows through a glass mixing coil. The uric acid from this stream passes through a dialyzer to a recipient stream containing hydroxylamine hydrochloride, and this stream is then mixed with sodium tungstate and phosphotungstic acid. The recipient stream is heated at 50–52°C, and the resultant blue color is measured at 660 nm in a flowcell on the sample side of a colorimeter.

The other half of the extract stream passes through a uricase coil before reaching its dialyzer. The uricase coil enzymatically destroys any uric acid that may be present. From this

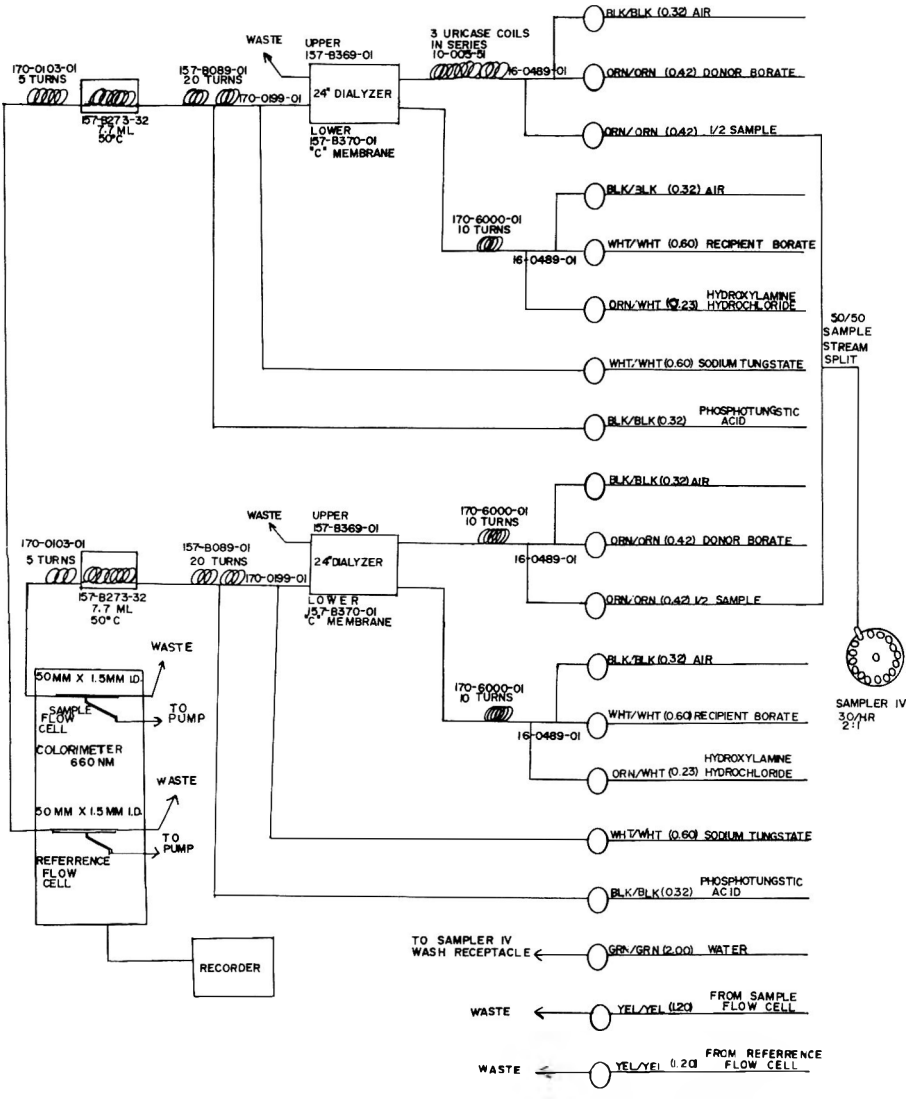

Figure 1. AutoAnalyzer system for determination of uric acid in flour extracts.

point on, the uricase-treated stream is treated the same as the other stream but passes through the reference side of the colorimeter after heating (see Roy and Kvenberg (7) for a more detailed description of the chemistry involved).

The split stream is synchronized to ensure that those portions of the stream which represent the same sample simultaneously pass through their respective flowcells. The resulting colorimeter signal (absorbance) is proportional to the uric acid content of the extract.

Results and Discussion

A mean recovery of 97.1% was obtained from eight 50 g bleached flour samples each spiked with 1.00 mg uric acid (Table 1). The recoveries were obtained from 4 different lots of flour on 4 different days. Recovery data of 0.50, 1.50, 2.00, and 3.00 mg spikes are also given in Table 1.

Colorimetric determinations of the extractions should be made the same day. Recoveries for 1.00 mg spikes were only 80% 24 h later, 60% 48 h later, and 25% after 72 h.

A standard concentration of 0.1 μg uric acid/mL deflects the recorder pen 2 scale divisions when the uricase coils are new and at peak efficiency. This translates to 25 μg/50 g flour and is defined as a trace amount. Twice this amount (50 μg/50 g flour) has been defined as the lowest measurable amount. The upper limit is 1.5–2.0

Table 1. Recovery of uric acid added to 50 g flour samples

Added, mg	*n*	Mean rec., %	Std dev.	Coeff. of var.	Range
0.50	2	101.1	6.36	6.33	105–96.0
1.00	8	97.1	6.51	6.71	110–89.0
1.50	2	107.0	14.1	13.2	117–97.0
2.00	2	69.0	9.19	13.3	75.5–62.5
3.00	2	46.8	7.35	15.7	52.0–41.6

mg/50 g flour (see Table 1) depending on the efficiency of the uricase coils. The coils gradually wear out but last around 6 months when in constant use.

A mixture of 1000 adult and larval fecal pellets obtained from *T. castaneum* raised on bleached flour was manually counted under a widefield microscope. Uric acid was extracted from these pellets using the method described by Roy and Kvenberg (7) for 1 g samples. The resulting 100 mL extraction solution represented a concentration of 10 pellets/mL. Further dilutions were made which resulted in solutions with concentrations representing 10, 8, 4, 2, and 1 pellet/mL. The mean uric acid value was 0.228 μg/pellet.

Twenty-three 50 g samples of flour were spiked with various numbers (200–1500) of excreta pellets from the same source. Twelve different lots of flour extracted on 5 different days were used. The mean recoveries ranged from 76.8% for 200 pellets to 117% for 1200 pellets (Table 2). The average mean recovery was 97.6% with an average standard deviation of 5.9 and an average coefficient of variation (CV) of 6.4%.

Forty-eight flour samples of known insect fragment count (IFC) were obtained from the Federal Grants Inspection Service (FGIS), U.S. Department of Agriculture, Beltsville, MD. The IFC ranged from 2 to 123 fragments/50 g flour. The samples represented 46 different lots of flour. The insect excreta pellets in five 1 g portions taken from each of the FGIS samples were counted on an extra large (11 × 9½ cm) glass counting plate, manufactured especially to adapt the official clove oil method (3) for use with 1 g samples. The total count of the 5 portions × 10 was used to obtain the number of insect pellets (IPs) per 50 g flour for each sample.

The resulting IP, IFC, and mean uric acid values for each of the FGIS samples are shown in Tables 3 and 4. The mean uric acid values were obtained from 2–5 replicate determinations. (The average percent CV was 8.10.) Table 3 ranks the samples in ascending order of IFC and Table 4 in ascending order of IP. Sample numbers were assigned according to ascending IFC order. Samples 26–48 would exceed the Food and Drug Administration's (FDA) current regulatory action guideline of 50 or more insect fragments/50 g flour.

The data in Table 3 show that as the IFC increases, IP generally also increases. Whenever the IP is notably smaller (Samples 9, 14, and 22) or larger (Samples 15, 18, 32, and 34) than the IPs of the immediately preceding and following samples (adjacent), the uric acid content is correspondingly smaller or larger for that sample.

The data in Table 4 also show that as IP increases, both the uric acid content and the IFC of a sample generally increase. Usually, whenever the IFC is significantly larger (Sample 28) or smaller (Samples 7 and 5) than the adjacent IFCs, the uric acid value for that sample is correspondingly larger or smaller than the adjacent sample uric acid values.

These data also demonstrate that *both* IFC and

Table 2. *T. castaneum* excreta pellet recoveries from 50 g flour, based on 0.228 μg uric acid/pellet

No. pellets added	*n*	Mean rec., %	Std dev.	Coeff. of var.	Range
200	2	76.8	15.2	19.8	87.5–66
400	4	95.9	1.85	1.04	97.5–94.3
500	3	104	6.88	6.62	110–96.4
800	4	98.1	4.40	4.48	104–93.8
1000	6	98.8	9.97	10.1	110–87.7
1200	2	117	0	0	117–117
1500	2	92.9	3.04	3.27	95.0–90.7

Table 3. Flour samples (50 g) ranked by insect fragment count

Below FDA guidelines				Exceed FDA guidelines			
Sample	IFC[a]	IP[b]	Uric acid, μg[c]	Sample	IFC[a]	IP[b]	Uric acid, μg[c]
1	2	50	39.5	26	54	270	620
2	2	180	214	27	57	490	471
3	4	140	277	28	59	140	631
4	4	150	471	29	62	440	466
5	5	280	196	30	62	660	779
6	6	130	480	31	63	330	956
7	7	250	51	32	65	1480	1016
8	9	240	152	33	66	690	541
9	10	100	53.5	34	66	1770	1043
10	24	200	710	35	68	700	572
11	29	320	535	36	68	350	557
12	32	220	698	37	69	1070	1054
13	33	340	618	38	69	430	738
14	33	190	trace[d]	39	69	740	861
15	35	670	702	40	72	650	565
16	36	250	391	41	78	800	1519
17	37	200	497	42	83	950	663
18	38	580	722	43	83	1470	679
19	41	430	578	44	88	890	842
20	41	320	447	45	103	3770	1434
21	44	240	529	46	104	2330	1601
22	45	150	294	47	122	2720	1375
23	45	250	616	48	123	1880	926
24	48	250	445				
25	48	330	773				

[a] IFC = insect fragment count.
[b] IP = number of insect excreta pellets.
[c] Mean of 2–5 replicates.
[d] Trace = less than 25 μg.

IP should be considered when uric acid is used to measure the degree of insect defilement. Therefore, ICF + IP = index of insect defilement.

Figure 2 is a plot of insect defilement vs uric acid using the data from Tables 3 and 4. The correlation coefficient (r) for the least squares straight line is 0.750. The relationship (plot) of IFC to uric acid is shown in Figure 3; $r = 0.731$. For 42 sample points with an "r" greater than 0.490, a valid relationship exists with >99.9% certainty. The correlation factors were calculated using only 42 points after discounting the 3 highest and 3 lowest uric acid values, since the highest and lowest values are the ones which strain the system the most. Therefore, it is greater than 99.9% certain that both insect defilement and IFC correlate with uric acid. Since insect defilement vs uric acid was the higher correlation coefficient, it has a slightly higher positive correlation.

As can be seen from Figures 2 and 3, if a cutoff value of 600 μg uric acid/50 g flour is used, only 6 of the tested samples which exceed FDA IFC

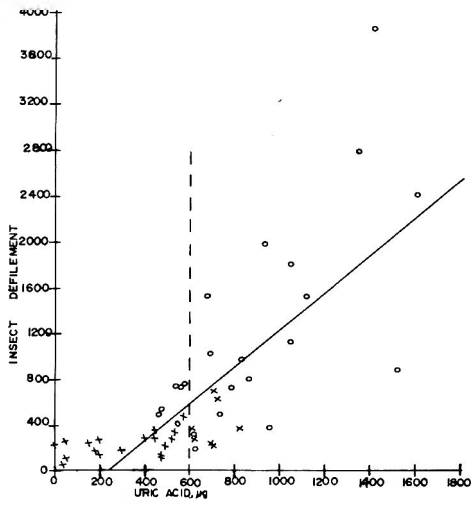

Figure 2. Correlation of insect defilement (insect fragments + insect excreta pellets) with uric acid content of 50 g samples of flour. 0 = 50 or more fragments; X = less than 50 fragments; – – – 600 μg uric acid/50 g flour cutoff line; —— least squares line.

Table 4. Flour samples (50 g) ranked by number of insect excreta pellets

Sample[a]	IP[b]	IFC[c]	Uric acid, μg[d]	Sample[a]	IP[b]	IFC[c]	Uric acid, μg[d]
1	50	2	39 5	36	350	68	557
9	100	10	53.5	19	430	41	578
6	130	6	480	38	430	69	738
3	140	4	277	29	440	62	466
28	140	59	631	27	490	57	471
4	150	4	471	18	580	38	722
22	150	45	294	40	650	72	565
2	180	2	214	30	660	62	779
14	190	33	trace[e]	15	670	35	702
10	200	24	710	33	690	66	541
17	200	37	497	35	700	68	572
12	220	32	698	39	740	69	861
8	240	9	152	41	800	78	1519
21	240	44	529	44	890	88	842
7	250	7	51	42	950	83	663
16	250	36	391	37	1070	69	1054
23	250	45	616	43	1470	83	679
24	250	48	445	32	1480	65	1016
26	270	54	620	34	1770	66	1043
5	280	5	196	48	1880	123	926
11	320	29	535	46	2330	104	1601
20	320	41	447	47	2720	122	1375
25	330	48	773	45	3770	103	1434
31	330	63	956				
13	340	33	618				

[a] Samples 26–48 exceed FDA's current guidelines.
[b] IP = number of insect excreta pellets.
[c] IFC = insect fragment count.
[d] Mean of 2–5 replicates.
[e] Trace = less than 25 μg.

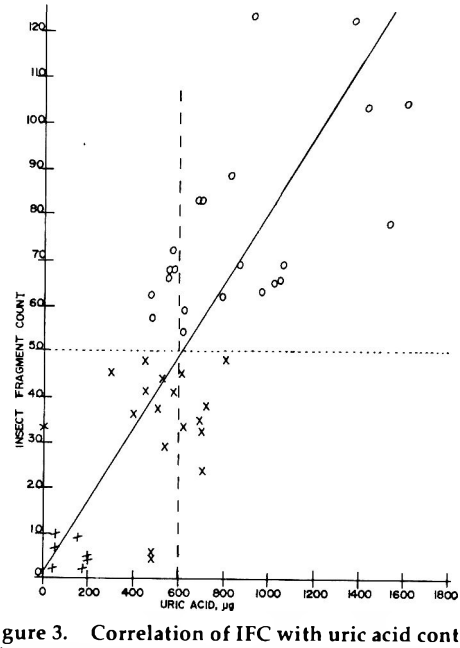

Figure 3. Correlation of IFC with uric acid content of 50 g samples of flour. 0 = 50 or more fragments; X = less than 50 fragments; - - - - 50 fragment/50 g flour cutoff line, — — — 600 μg uric acid/50 g flour cutoff line; —— least squares line.

guidelines would not be considered violative and 7 of the samples which are below FDA IFC guidelines would be violative. All the data points on the wrong side of the line could be considered to lie in the borderline area. Also, these data points are based on only 1 IFC for each lot of flour, while the FDA guideline is based on an average of 6 IFCs per lot.

Some of the other apparent poorer correlations in Figures 2 and 3 originate from the documented phenomenon that insects of different species produce different amounts of uric acid (5–11). Several different species of insects are known to infest flour (5, 7, 11) and the specific sources of the fragments and pellets present in the naturally contaminated samples were not determined.

Conclusions

The most accurate measurement of the degree to which flour has been defiled includes both the number of insect excreta pellets and the number of insect fragments present. A valid relationship has been shown to exist between the uric acid content of a flour sample and the number of insect excreta pellets and insect fragments in it

(Figure 2). Therefore, the measurement of the uric acid content of flour can be used as a valid index of insect defilement.

A valid relationship has also been demonstrated between the IFC of a sample and its uric acid content (Figure 3). Therefore, the IFC is a valid method of determining insect defilement.

The results show that the proposed method could be used as a screening procedure for determining the amount of insect defilement in flour. The data indicate that a uric acid value of 600 μg/50 g flour approximates the same degree of insect defilement as FDA's current guideline of 50 insect fragments/50 g flour and suggest that samples exceeding 600 μg would contain violative amounts of insect filth.

The proposed method offers the advantages of improved speed (16 samples may be completed in 1 day as compared to 8 samples by the official microscopic method); increased uric acid sensitivity, which enables chemical measurements below the current FDA insect filth in flour guideline; large sample size (50 g), which enables analysis of a representative amount of the sample; and a chemical determinative step, which eliminates the need for the analyst to have entomological expertise. It is suggested that this method be subjected to a collaborative study.

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FOOD ADDITIVES

Ion-Pair High Performance Liquid Chromatographic Determination of Inosinic Acid in Meat

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An ion-pair high performance liquid chromatographic (HPLC) technique is described for the determination of inosinic acid (IMP) in meat. The compound was extracted with perchloric acid and analyzed without cleanup. IMP is effectively separated, identified, and quantitated by using a reverse phase column, with ultraviolet detection. A C_8 stationary phase and tetrabutyl ammonium as counter ion are used. Recovery of IMP added to meat at 500 or 2500 ppm levels was more than 95%; the limit of detection for IMP is 50 ppm.

Inosinic acid (IMP) is a type of monoribonucleotide and often appears in natural foods such as dried fish, beef, and chicken meat (1). In Japan, IMP has been registered as a food additive since 1960, but there is no established tolerance for food type and volume. We wished to develop a rapid, accurate method for extraction and quantitation of IMP in meat, because it plays an important role in meat quality.

Several high performance liquid chromatographic (HPLC) methods have been shown to be useful for determining nucleotides, nucleosides, and free bases. They have been based on ion-exchange (2–7) and reverse phase column chromatography (8–10). The proposed method for determining IMP in meat involves extraction with perchloric acid; no concentration or prior cleanup is needed. The determination was carried out by a reverse phase HPLC system with a C_{18} column to assure identification of IMP and other monoribonucleotides.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Shimadzu Model LC-2 (Shimadzu Seisakusho Ltd, Kyoto, Japan).

(b) *Column.*—Column A: stainless steel, 4 mm id × 25 cm, with LiChrosorb RP-8, 7 μm (E. Merck, Darmstadt, GFR). Column B: stainless steel, 4.6 mm id × 15 cm, with Zorbax ODS, 5–6 μm (Du Pont, Wilmington, DE 19898). Guard column: stainless steel, 4 mm id × 5 cm, dry-packed (tap and fill method) with Bondapak C_{18}, Corasil, 37–50 μm (Waters Associates, Milford, MA).

(c) *Detector.*—Shimadzu Model UVD-2 ultraviolet monitor (Shimadzu Seisakusho Ltd).

(d) *High-speed blender.*—Nihon Seiki Model AM-7 equipped with stainless steel cup (Nihon Seiki Ltd, Tokyo, Japan).

(e) *Sample filtering system.*—25 mm filter holder and Fluoropore filters, 1.0 μm pore size (Sumitomo Electric Industry Ltd, Osaka, Japan); 47 mm filter holder and membrane filters, 0.22 μm pore size (Millipore Corp., Bedford, MA); No. 5C filter paper (Toyo Roshi Ltd, Tokyo, Japan).

(f) *Centrifuge.*—Hitachi Model 18PR-3 (Hitachi Seisakusho Ltd, Tokyo, Japan).

(g) *Shaker.*—Iwaki Model V-D (Iwaki Co. Ltd, Tokyo, Japan).

Reagents

(a) *Chemicals.*—Inosine-5′-phosphate, sodium salt (IMP); cytidine-5′-phosphate, sodium salt (CMP); uridine-5′-phosphate, sodium salt (UMP); and guanosine-5′-phosphate, sodium salt (GMP) were obtained from P-L Biochemicals, Inc., Milwaukee, WI 53205. Adenosine-5′-phosphate, sodium salt (AMP); tetra-*n*-butyl ammonium bromide (TBA); methanol; 60% perchloric acid; and other chemicals were obtained from Wako Pure Chemical Industry Ltd, Osaka, Japan.

(b) *Standard solution.*—500 μg/mL. Dissolve 0.125 g IMP in 250 mL pH 7.3 phosphate buffer prepared according to method of Al-Moslih et al. (10).

(c) *Mobile phase.*—Phase A: pH 8.0 phosphate buffer + 5 mM TBA. Dissolve 9.929 g $Na_2HPO_4 \cdot 12H_2O$, 0.308 g KH_2PO_4, and 1.612 g TBA in water and dilute to 1 L. Phase B: pH 5.8 phosphate buffer. Dissolve 1.158 g $Na_2HPO_4 \cdot 12H_2O$ and 6.360 g KH_2PO_4 in water and dilute to 1 L. Filter mobile phases through 0.22 μm membrane filters.

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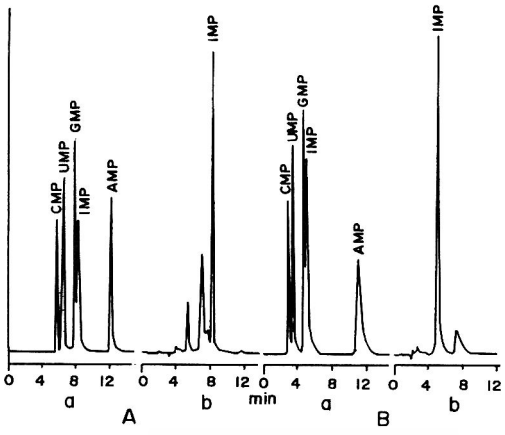

Figure 1. Liquid chromatograms of standard ribonucleotides and pork extract: A, column A conditions; B, column B conditions. a, standard ribonucleotides (100 μg/mL, respectively); b, pork sample (0.1 g/mL) containing 0.500 μg standard, 0.875 μg IMP in pork extract.

Preparation of Sample Extract

Use beef, pork, and chicken meat. Finely slice ca 100 g meat, weigh 10 g mixture into 100 mL high-speed blender cup, add 40 mL aqueous 5% perchloric acid, and extract by homogenizing 3 min at 15 000 rpm (28 400 × *g*). Transfer homogenate to 70 mL centrifuge tube. Wash blender cup and cutter with 10 mL 5% perchloric acid, transfer washing to centrifuge tube, and centrifuge at 10 000 rpm (12 600 × *g*) for 5 min. Filter supernate into 200 mL glass beaker through No. 5C paper by suction. Add 20 mL 5% perchloric acid to centrifuge tube, shake 5 min, centrifuge, and filter supernate into beaker. Adjust pH of combined filtrate to 6.5 with 5N NaOH and 0.5N NaOH. Transfer filtrate to 100 mL volumetric flask, dilute to volume with water, and mix before filtration. Then filter through 1.0 μm filter.

High Performance Liquid Chromatography

Dilute standard solution to prepare 50, 100, 150, 200, 250, and 300 μg IMP/mL. Inject 5 μL of each standard solution into chromatograph. Set column temperature at 40°C. Adjust flow rate to 0.9 mL/min. Prepare standard curve by plotting peak height of IMP vs concentration. Run sample extracts under same conditions used for standards. Read amount (μg/mL) of IMP in extract from standard curve. Multiply this amount by 10 as a dilution factor (100 mL extract/10 g sample meat) to obtain concentration (μg/g) of IMP in sample meat.

Table 1. Recovery of inosinic acid added to meats [a]

	500 ppm			2500 ppm		
Sample	Rec., %	SD	CV, %	Rec.,%	SD	CV, %
Beef	96.2	2.2	2.26	98.5	1.0	0.97
Pork	95.8	2.6	2.71	99.6	1.1	1.11
Chicken	94.9	2.1	2.20	97.9	0.8	0.84

[a] Results of 5 trials.

Results and Discussion

Kusuwi et al. (6) extracted IMP from fish paste product, soy sauce, granule bouillon, etc., with 5% perchloric acid. We extracted IMP from meat according to their method.

Column A was used for the determination of IMP. We also tried to determine the presence of CMP, UMP, GMP, and AMP in meat. But in column A, determination of CMP and GMP in pork, CMP and UMP in beef, and CMP in chicken meat had interferences by natural meat components. Whereas in column B, GMP had interferences by natural chicken meat components. Therefore, it was necessary to use 2 HPLC columns to assure identification of 5 monoribonucleotides. As a result, those ribonucleotides were less than 50 ppm. In column A, GMP and IMP were not separated at the baseline without TBA, and it was necessary to adjust the pH of the mobile phase to 8.5 to obtain better baseline separation of 5 ribonucleotides. No peaks that interfere with the determination of IMP have been observed in tested meats. Figure 1 shows typical standard and sample extract chromatograms by columns A and B.

Table 1 shows the recoveries from meats fortified at 500 or 2500 ppm. Added IMP was recovered in the range 94.9–99.6%. We were able to detect 5 μg IMP/mL by the method, so the detection limit was 50 ppm when this amount was multiplied by a dilution factor.

Acknowledgments

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ANTIBIOTICS

Microbiological Assay for Antibiotics in Surface Waters

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Assay procedures were developed for determining bacitracin, chlortetracycline, erythromycin, oxytetracycline, penicillin, streptomycin, and tylosin residues in surface waters. Direct addition of solid potassium phosphate buffering salts to water samples enabled suitable pH adjustments for optimum assay conditions. Recoveries from 2 surface waters averaged 97.9 and 102.7% for chlortetracycline, 101.0 and 101.5% for bacitracin, 94.5 and 95.3% for erythromycin, 84.2 and 89.8% for oxytetracycline, 82.3 and 97.5% for penicillin, 97.4 and 99.2% for streptomycin, 87.4 and 94.2% for tylosin.

The FDA Environmental Impact Statement (1), perhaps the most definitive review of the literature concerning the fate of antibacterial agents used in animal feeds, noted the paucity of information and data as well as the large amount of speculation related to the stability of antibiotics in the environment. Although some data exist relative to the stability of antibiotics in soil, little is known concerning their stability in surface waters.

Feinman and Matheson (1) felt that the tetracyclines, bacitracin, tylosin, and erythromycin were mobile in soil, depending on the soil clay content; penicillin was possibly mobile, while streptomycin and neomycin would be minimally mobile. However, they did not speculate on the overall potential of movement of antibiotics from animal wastes to soils and eventually to surface waters.

Some evidence exists that antimicrobials can be leached from pastures into streams. Van Dijck and Van de Voorde (2) reported that 12 of 41 stream water samples taken in an agricultural area showed inhibitory activity. No data were presented identifying the sources or the moieties causing the inhibitory activity. Rumsey et al. (3) noted that trace quantities of chlortetracycline were found at times in runoff from pastures on which wastes containing the antibiotic had been spread.

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The purpose of this paper is to present simple, sensitive, and reproducible procedures that can be used to determine the levels of antibiotics in surface waters. These procedures can provide the methodological basis for studies on the stability of antibiotics in surface waters.

METHOD

Nutrient Media

(a) *Antibiotic medium No. 1.*—For bacitracin, tylosin, and penicillin. (Medium manufactured by Scott Laboratories was satisfactory.)

(b) *Antibiotic medium No. 5.*—For streptomycin; medium No. 8, low pH, for the tetracyclines; medium No. 11, for erythromycin. (Media manufactured by BBL were satisfactory.)

Buffers

All buffer salts are dissolved and diluted to 1 L with deionized water.

(a) *Phosphate buffer, pH 4.5.*—For tetracyclines. Contains 13.6 g monopotassium phosphate.

(b) *Phosphate buffer, pH 6.0.*—For penicillin. Contains 8.0 g monopotassium phosphate and 2.0 g dipotassium phosphate.

(c) *Phosphate buffer, pH 6.5.*—For bacitracin. Contains 28.0 g monopotassium phosphate and 22.0 g dipotassium phosphate.

(d) *Phosphate buffer, pH 8.0.*—For tylosin and erythromycin. Contains 16.73 g dipotassium phosphate and 0.523 g monopotassium phosphate.

Test Microorganisms

All organisms can be prepared according to procedures described by AOAC (4) or Kramer et al. (5).

(a) *Bacillus cereus var. mycoides ATCC 11778.*—For tetracyclines.

(b) *Bacillus subtilis ATCC 6633.*—For streptomycin.

(c) *Micrococcus luteus (Sarcina lutea) ATCC 9341.*—For penicillin, erythromycin, and tylosin.

(d) *Micrococcus luteus (Micrococcus flavus) ATCC 10240.*—For bacitracin.

Preparation of Plates

Use plastic petri dishes (15 × 100 mm). For chlortetracycline, oxytetracycline, and streptomycin assays, cool designated media to 65°C and inoculate with appropriate spore suspension. For penicillin, tylosin, erythromycin, and bacitracin, cool specified media to 48°C and inoculate with indicated organism. Evenly spread 7 mL inoculated assay agar in each dish and let cool with covers ajar to let condensation escape. Place 6 cylinders on each plate, using Shaw or equivalent automatic cylinder dispenser according to AOAC procedures (4).

Preparation of Standard Curve

Prepare standard solutions containing 100 μg or 100 units activity/mL as described by AOAC procedures (4). For chlortetracycline and penicillin, standard curve range is 0.01, 0.02, 0.04, 0.08, and 0.16 μg/mL with 0.04 μg/mL as reference concentration; for oxytetracycline, bacitracin, and erythromycin, range is 0.025, 0.05, 0.10, 0.20, 0.40, and 0.80 μg/mL with 0.20 μg/mL as reference concentration; for tylosin and streptomycin, range is 0.125, 0.25, 0.50, 1.00, and 2.00 μg/mL with 0.50 μg/mL as reference concentration.

Measurement of Potency

Use 3 petri dishes for each sample. On each plate, fill 3 alternate cylinders with reference concentration and 3 with sample assay solution. Incubate plates 18–24 h at 30°C. After incubation, remove cylinders, gently wash surface of plates, and measure zones of inhibition with Fisher-Lilly zone reader. Determine potency of samples according to AOAC procedures (4).

Response of Test Microorganisms

For proper sensitivity, test organisms should give following response to defined concentrations of antibiotic:

Antibiotic	*Concn, unit or μg/mL*	*Expected response, mm*
Bacitracin	0.10	13.5–14.5
Chlortetracycline	0.01	14.0–15.0
Erythromycin	0.05	16.5–17.5
Oxytetracycline	0.05	12.0–13.0
Penicillin	0.02	13.5–14.5
Streptomycin	0.25	15.0–16.0
Tylosin	0.25	12.0–13.0

Assay of Residues in Surface Waters

Collection: Collect representative surface waters on same day of analysis. Collect samples by placing clean, but not sterile, 1 gal. bottles ca 6 in. below surface. Return samples to laboratory immediately (usually within 15 min) and use for supplementation studies within 1 h.

Supplementation: Add enough antibiotic activity, derived from standard solutions, to 1 L volumetric flasks to yield concentrations equivalent to those of standard response line when representative surface water is added to flask and flask is brought to volume. Mix well and let supplemented water equilibrate 1 h.

Assay for antibiotic activity: According to following schedule, add designated quantities of solid phosphate salts and/or volumes of aqueous salts to 250 mL Erlenmeyer flasks.

Antibiotic	*KH_2PO_4, g*	*K_2HPO_4, g*	*0.0523 g KH_2PO_4/mL, mL*	*Expected pH range, assay soln*
Chlortetracycline	1.36	—	—	4.5–4.7
Oxytetracycline	1.36	—	—	4.5–4.7
Streptomycin	—	1.67	1.0	8.0 ± 0.05
Tylosin	—	1.67	1.0	8.0 ± 0.05
Erythromycin	—	1.67	1.0	8.0 ± 0.05
Bacitracin	2.8	2.2	—	6.2–6.5
Penicillin	0.8	0.2	—	6.0–7.0

Add 100 mL aliquots from volumetric flasks and mix well to dissolve phosphate buffer. Adjust pH if necessary. These are the assay solutions.

Results and Discussion

Sensitive analytical procedures are needed for determining antibiotic residues in the environment. No organized procedures are available for determining antibiotic residues in surface waters. The lack of definitive information concerning the fate of antibiotics in the environment requires such methodology as a first step in developing an organized body of knowledge. The aquatic medium is a matrix that allows extremely sensitive procedures. Unlike microbiological assay procedures for determining antibiotic residues in milk, animal muscle, and organ tissue where extraction, dilution, and sensitivity problems exist, the procedures reported herein

are relatively simple and free from the aforementioned problems.

Two sources of water were used in these studies: College Farm Pond at Cook College, and Farrington Lake, East Brunswick, NJ. College Farm Pond is a typical farm pond filled by runoff and drainage from surrounding lawns and fields. Farrington Lake is a long, narrow lake which receives drainage from several tributary creeks. These creeks pass through an area of truck and animal farms. Both sources represent the type of surface waters where antibiotics used for animal agriculture might be found because of leaching from wastes or from pastures used for disposal of wastes. Both sources of water possess a fair degree of suspended matter, have a high bacterial population, and have a pH ranging from 6.2 to 6.6. College Farm Pond also contains a high algal population.

The use of the buffering salts dipotassium hydrogen phosphate and potassium dihydrogen phosphate in the dry state, added directly to the water sample, provides the proper buffering with no need for dilution. Although the natural buffering capacity of the surface water may cause some minor deviations from the anticipated optimum assay pH for a given antibiotic, final pH adjustment can be accomplished easily with little change in sample volume.

The sensitivity of detection and measurement for individual antibiotics was similar if not identical to that obtained in buffer systems used for standard response lines. Table 1 shows recoveries obtained for chlortetracycline and penicillin assayed in the representative surface waters. Recoveries of chlortetracycline were essentially quantitative, averaging 97.9% in water from College Farm Pond and 102.7% in water from Farrington Lake. Recoveries of penicillin averaged 82.3% in water from the farm pond and 97.5% in water from the lake. Recoveries of chlortetracycline were not affected by differences in suspended matter, but penicillin recoveries did appear to be affected. The absorbance of the water, measured at 580 nm (a gross measurement of suspended material), was 0.140 for College Farm Pond and 0.025 for Farrington Lake. Although this is a crude estimate of bacteria, algae, clay, silt, detritus, and similar materials, it gives some insight to factors that might affect recovery. Penicillin recoveries might have been affected by the presence of increased suspended matter; the higher recoveries were obtained in the water with the lesser amount of turbidity.

To estimate the variability of the procedure, chlortetracycline was added to the surface waters at 3 concentrations, 0.01, 0.04, and 0.08 μg/mL. Determinations were replicated 5 times. Table 2 shows the recoveries and range of recoveries obtained from this methodology. Considering the levels studied, repeatability was adequate. Although data for chlortetracycline were used to estimate accuracy and precision of the methodology, perusal of the data obtained for the other antibiotics indicates similar recovery patterns.

Table 3 shows the recoveries of oxytetracycline, erythromycin, and bacitracin from both surface waters. Recoveries of bacitracin were the highest, averaging slightly over 100%; erythromycin recoveries averaged 95%, and oxytetracycline recoveries averaged 87%. Recoveries of bacitracin exhibited the greatest positive bias with the largest number of samples exceeding 100%. Table 4 shows the recoveries of tylosin and streptomycin. Tylosin recoveries were slightly lower in the water from College Farm Pond than in the lake water, an average of 87.4% vs 94.2%. Recoveries of streptomycin from both water sources showed little difference, although streptomycin, like bacitracin, showed a number of recoveries above 100%.

Average recoveries obtained for the 2 tetra-

Table 1. Recoveries of chlortetracycline and penicillin from representative surface waters

Sample concn, unit or μg/mL	Chlortetracycline		Penicillin	
	Found, μg/mL	Rec., %	Found, unit/mL	Rec., %
College Farm Pond				
Blank	ND[a]	—	ND	—
0.005	0.005	100.0	NP[b]	—
0.010	0.010	100.0	0.008	80.0
0.020	0.020	100.0	0.016	80.0
0.040	0.039	97.5	0.031	77.5
0.080	0.077	96.2	0.074	92.5
0.160	0.150	93.8	0.130	81.3
Av.	—	97.9	—	82.3
Farrington Lake				
Blank	ND	—	ND	—
0.005	0.005	100.0	NP	—
0.010	0.011	110.0	0.009	90.0
0.020	0.023	115.0	0.020	100.0
0.040	0.040	100.0	0.034	85.0
0.080	0.078	97.5	0.085	106.3
0.160	0.150	93.8	0.170	106.2
Av.	—	102.7	—	97.5

[a] None detected.
[b] Not performed.

Table 2. Repeatability of assay system, using chlortetracycline as representative antibiotic [a]

Added, μg/mL	College Farm Pond		Farrington Lake	
	Found, μg/mL	Rec., %	Found, μg/mL	Rec., %
0.010	0.009	90.0	0.008	82.0
	0.009	90.0	0.0098	98.0
	0.0084	84.0	0.0078	78.0
	0.0100	100.0	0.0086	86.0
	0.0084	84.0	0.0060	60.0
Av.	0.0089	89.6	0.0081	80.8
SD	0.007	5.5	0.0012	12.4
Range [b]	0.0089 ± 0.0006	89.6 ± 5.2	0.0081 ± 0.001	80.8 ± 10.8
0.040	0.037	92.5	0.037	92.5
	0.036	90.0	0.040	100.0
	0.038	95.0	0.038	95.0
	0.038	95.0	0.035	87.5
	0.036	90.0	0.040	100.0
Av.	0.037	92.5	0.038	95.0
SD	0.001	2.2	0.008	4.7
Range	0.037 ± 0.001	92.5 ± 2.0	0.038 ± 0.003	95.0 ± 4.1
0.080	0.076	95.0	0.076	95.0
	0.072	90.0	0.072	90.0
	0.072	90.0	0.080	100.0
	0.070	87.5	0.072	90.0
	0.082	102.5	0.062	77.5
Av.	0.074	93.0	0.072	90.5
SD	0.004	5.3	0.006	7.5
Range	0.074 ± 0.003	93.0 ± 4.6	0.072 ± 0.005	90.5 ± 6.6

[a] Each value is average of 5 replicates.
[b] 95% confidence limits.

Table 3. Recoveries of oxytetracycline, erythromycin, and bacitracin from representative surface waters

Sample concn, unit or μg/mL	Oxytetracycline		Erythromycin		Bacitracin	
	Found, μg/mL	Rec., %	Found, μg/mL	Rec., %	Found, unit/mL	Rec., %
			College Farm Pond			
Blank	ND [a]	—	ND	—	ND	—
0.025	0.025	100.0	0.026	104.0	NP [b]	—
0.050	0.042	84.0	0.044	88.0	0.047	94.0
0.100	0.080	80.0	0.080	80.0	0.091	91.0
0.200	0.180	90.0	0.190	95.0	0.180	90.0
0.400	0.340	85.0	0.400	100.0	0.430	107.5
0.800	0.800	100.0	0.800	100.0	1.000	125.0
Av.	—	89.8	—	94.5	—	101.5
			Farrington Lake			
Blank	ND	—	ND	—	ND	—
0.025	0.022	88.0	0.026	104.0	NP	—
0.050	0.036	72.0	0.044	88.0	0.042	84.0
0.100	0.085	85.0	0.080	80.0	0.110	110.0
0.200	0.180	90.0	0.200	100.0	0.210	105.0
0.400	0.360	90.0	0.400	100.0	0.400	100.0
0.800	0.640	80.0	0.800	100.0	0.850	106.2
Av.	—	84.2	—	95.3	—	101.0

[a] None detected.
[b] Not performed.

Table 4. Recoveries of tylosin and streptomycin from representative surface waters

Sample concn, μg/mL	Tylosin		Streptomycin	
	Found, μg/mL	Rec., %	Found, μg/mL	Rec., %
College Farm Pond				
Blank	ND[a]	—	ND	—
0.125	0.125	100.0	0.15	120.0
0.25	0.23	92.0	0.19	76.0
0.50	0.40	80.0	0.54	108.0
1.00	0.90	90.0	1.02	102.0
2.00	1.50	75.0	1.80	90.0
Av.	—	87.4	—	99.2
Farrington Lake				
Blank	ND	—	ND	—
0.125	0.110	88.0	0.120	96.0
0.25	0.25	100.0	0.22	88.0
0.50	0.44	88.0	0.54	108.0
1.00	0.95	95.0	1.05	105.0
2.00	2.00	100.0	1.80	90.0
Av.	—	94.2	—	97.4

[a] None detected.

cyclines differed at about 10–20%; the higher recoveries were obtained for chlortetracycline. The reason for this is unknown but it is not related to a greater amount of suspended matter in the water. The recoveries of the 2 macrolide antibiotics, erythromycin and tylosin, were similar and no special note should be made of the differences.

Of the antibiotics studied, the tetracyclines, the macrolides, and bacitracin are the most likely to appear in surface waters. Penicillin is degraded quickly in soil and excreta, and the possibility of penicillin leaching into surface water is minimal. Streptomycin is tightly bound by clays in the soil and hence its mobility is, at best, minimal. The solubility and minimal reactivity of the tetracyclines, the macrolides erythromycin and tylosin, and bacitracin with clays in the soil indicate a potential for leach. All antibiotics can appear in surface waters adjacent to manufacturing facilities because of inappropriate disposal methods.

The approach advanced in this manuscript should be extendable to determining other antibiotics in surface waters. However, a qualitative scheme of analysis to support the quantitative determinations is sorely needed. Methodology combining both qualitative and quantitative systems would provide the tools to study the occurrence and stability of antibiotics in surface waters.

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Microbial Assay Systems for Determining Antibiotic Residues in Soils

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Procedures for the assay of bacitracin, chlortetracycline, erythromycin, oxytetracycline, and tylosin residues in soils are presented. Except for tylosin, 1 μg antibiotic/g soil could be detected and measured. Recoveries of bacitracin (43.0-83.9%) averaged 60.3% and were unaffected by clay, sand, or silt content. Recoveries of chlortetracycline (40.0-116.0%) averaged 63.9% with no apparent relationship between recoveries and soil composition. Recoveries of oxytetracycline (30.8-100.0%) averaged 64.5% and were affected by silt and clay content. There was an inverse relationship between recoveries of tylosin and clay and silt content. Recoveries ranged from 6.5% in a soil containing 23.7% silt and 16.3% clay to 74.1% in a soil containing 96.3% sand. Recoveries of erythromycin were similarly related to clay and silt content, with recoveries averaging 36.3% in soil containing 40% silt and clay and 76.0% in soil containing 96.3% sand. Penicillin could not be recovered from soils because of its instability. Streptomycin was irreversibly adsorbed and could not be extracted from any soil studied.

The stability in and transport through soil, and overall chemical and biological reactivity of antibiotics incorporated into soil as part of animal agricultural practice remains, at best, partially understood. Pinck et al. (1-5) studied the physicochemical aspects of antibiotics in soil. Jeffreys (6) studied the stability of several antibiotics in different soils and noted that all antibiotics studied "exhibited a fair degree of stability in some of the soils," although the rates of inactivation of the various antibiotics varied from soil to soil. The main interest in the aforementioned studies was related to the production/occurrence and stability of antibiotics in soil rather than the degradation of antibiotics resulting from agricultural uses. Similar considerations were the basis of the studies of Pramer and Starkey (7, 8), concerning the stability of streptomycin in soils.

The wastes resulting from animals fed antibiotics, regardless of whether the animals were managed in a feed-lot operation or on farms, invariably will find their way onto/into the soil and, potentially via transport and run-off, into surface waters. Elmund et al. (9) reported several general problems associated with the decomposition of feed-lot wastes containing chlortetracycline and raised the potential problem of waste-contaminated run-off water. The overall assessment in the Environmental Impact Statement of the FDA (10) vis-a-vis antimicrobial agents fed to animals and their fate in the environment indicated a lack of definitive knowledge concerning the measurement and the fate of antibiotics in soils.

The methodology available in the literature for the assay of antibiotics in soils is both unorganized and poorly described. Soulides et al. (3) used various buffer extraction procedures at specific pH values for a direct assay. Soulides (5) also used a combination of phosphate buffer extraction, evaporation of the buffer, re-extraction with solvents to remove salts, evaporation of the solvent, and resolubilization of the antibiotics in buffers followed by microbiological assay. The direct procedure was insensitive while the extraction-isolation techniques yielded fairly sensitive detection. The extraction-isolation procedure was long and tedious and subject to interferences if the solvent removal was not performed carefully. Elmund et al. (9) used AOAC methodology for chlortetracycline in feeds and reported reasonably good success. Jeffreys (6) used buffers for extraction but the levels detected were quite high.

The purpose of this report is to present a more organized body of extraction procedures coupled with microbiological assays which could measure some representative antibiotics in soils with a fair combination of sensitivity and accuracy. A level of 1 μg or unit/g was established because it is doubtful that concentrations below this level would have any effect on the development of resistance or would interfere with the usual functions performed by soil microorganisms (J. Gavalachin, Cook College, Rutgers University, private communication (1982)).

METHOD

Nutrient Media

(a) *Medium No. 1 for bacitracin, tylosin, and penicillin.*—Medium No. 1 manufactured by Scott Laboratories was satisfactory.

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(b) *Medium No. 5 for streptomycin, medium No. 8, low pH for the tetracyclines, and medium No. 11 for erythromycin.*—These media manufactured by BBL were satisfactory.

Buffers

Dissolve and dilute all buffer salts with deionized water to 1 L.

(a) *Phosphate buffer, pH 4.5, for the tetracyclines.*—Contains 13.6 g monopotassium phosphate.

(b) *Phosphate buffer, pH 6.0, for penicillin.*—Contains 8.0 g monopotassium phosphate, 2.0 g dipotassium phosphate.

(c) *Phosphate buffer, pH 6.5, for bacitracin.*—Contains 28.0 g monopotassium phosphate and 22.0 g dipotassium phosphate.

(d) *Phosphate buffer, pH 8.0, for tylosin, erythromycin, streptomycin.*—Contains 16.73 g dipotassium phosphate and 0.523 g monopotassium phosphate.

Assay Microorganisms

(a) *Bacillus cereus var. mycoides ATCC 11778 for tetracyclines.*

(b) *Bacillus subtilis ATCC 6633 for streptomycin.*

(c) *Micrococcus luteus (Sarcina lutea) ATCC 9341 for erythromycin, penicillin, and tylosin.*

(d) *Micrococcus luteus (Micrococcus flavus) ATCC 10240 for bacitracin.* Prepare all nutrient media, buffers, and assay organisms according to procedures of AOAC (11).

Preparation of Plates

Use plastic petri dishes (15 × 100 mm). For chlortetracycline, oxytetracycline, and streptomycin assays, cool designated medium to 65–70°C and inoculate with the appropriate level of indicated spore suspensions. For penicillin, tylosin, erythromycin, and bacitracin assays, cool specific medium to 48°C and inoculate with indicated organism. Evenly spread 7 mL inoculated assay agar in each dish. Let media in plates cool with covers ajar to permit escape of condensation. Place 6 cylinders on each plate, using Shaw or equivalent cylinder dispenser.

Preparation of Standard Curve

Prepare standard solutions containing 100 μg or 100 units activity/mL as described by AOAC (11). For chlortetracycline and penicillin, concentrations of standard response line are 0.01, 0.02, 0.04, 0.08, and 0.16 μg or u/mL with 0.04 μg or u/mL as reference concentration. For oxytetracycline, bacitracin, and erythromycin, concentrations are 0.025, 0.05, 0.10, 0.20, 0.40, 0.80 μg or u/mL with 0.20 μg or u/mL as reference concentration. For tylosin and streptomycin, concentrations are 0.125, 0.25, 0.50, 1.00, and 2.00 μg or u/mL with 0.50 μg or u/mL as reference concentration. Dilute tetracycline preparations in pH 4.5 phosphate buffer, penicillin in pH 6.0 buffer, bacitracin in pH 6.5 buffer, erythromycin, streptomycin, and tylosin in pH 8.0 buffer.

Measurement of Potency

Use 3 petri dishes for each sample. Fill 3 alternate cylinders with reference concentration and 3 alternate cylinders with sample assay solution. Incubate all assays at 30°C. After incubation, remove cylinders, wash surfaces of plates gently, measure zones of inhibition with Fisher-Lilly zone reader, and determine potency of samples as described by AOAC (11).

Response of Assay Microorganism

Assay organisms should yield the following zones of inhibition in response to the following concentrations of antibiotics:

Antibiotic	*Concentration, u or μg/mL*	*Response, mm*
Bacitracin	0.10	13.5–14.5
Chlortetracycline	0.01	14.0–15.0
Erythromycin	0.05	16.5–17.5
Oxytetracycline	0.05	12.0–13.0
Penicillin	0.02	13.5–14.5
Streptomycin	0.25	15.0–16.0
Tylosin	0.25	12.0–13.0

Assay of Antibiotics in Soils

Supplementation

Weigh 8.0 g dry soil into 250 mL Erlenmeyer flask. Add appropriate amount of antibiotic dissolved in deionized water so that final weight is not greater than 10.0 g. Let soil and antibiotic equilibrate 1 h.

Extracting Solvents

(a) *Bacitracin.*—Methanol–pH 6.5 phosphate buffer (75 + 25).

(b) *Erythromycin.*—Methanol–pH 8.0 phosphate buffer (50 + 50).

(c) *Tetracyclines.*—Methanol–conc. HCl (80 + 20).

(d) *Tylosin.*—pH 8.0 phosphate buffer.

(e) *Penicillin.*—Acetone–pH 6.0 phosphate buffer (50 + 50).

(f) *Streptomycin.*—0.5N HCl.

Procedure

(a) *Bacitracin.*—Take 10 mL aliquot and dilute to 50 mL with pH 6.5 buffer. Assay against standards prepared in methanol–pH 6.5 buffer (15 + 85).

(b) *Chlortetracycline.*—Remove 10 mL aliquot and dilute to ca 75 mL with pH 4.5 buffer. Carefully adjust pH to 4.5 with 3N potassium hydroxide. Dilute to 100 mL with pH 4.5 buffer. Assay using standards prepared in pH 4.5 buffer containing 8% methanol.

(c) *Oxytetracycline.*—Take 20 mL aliquot and follow procedure for chlortetracycline. Assay against standards prepared in pH 4.5 buffer containing 16% methanol.

(d) *Erythromycin.*—Remove 15 mL aliquot and dilute to 100 mL with pH 8.0 phosphate buffer. Assay against standards prepared in pH 8.0 buffer containing 7.5% methanol.

(e) *Tylosin.*—Adjust pH of extract to 8.0, if necessary. Use directly as assay solution. Assay against standards prepared in pH 8.0 buffer.

(f) *Penicillin.*—Take 10 mL aliquot and dilute to 100 mL with pH 6.0 buffer. Assay against standards prepared in pH 6.0 buffer containing 5% acetone.

(g) *Streptomycin.*—Remove 20 mL aliquot and dilute to ca 70 mL with pH 8.0 buffer. Adjust pH to 8.0 with 3.0N KOH. Dilute to 100 mL with pH 8.0 buffer. Assay against standards prepared in pH 8.0 buffer.

Soils

The soils chosen varied considerably in clay, silt, and sand content. The following summarizes the character of the soils used:

Soil	*Clay, %*	*Silt, %*	*Sand, %*
Adelphia sandy loam	16.3	23.7	60.0
Suburban garden soil	10.5	9.4	80.1
Agricultural sandy soil	3.4	0.3	96.3

Results and Discussion

The 1978 Environmental Impact Statement on the use of subtherapeutic antibacterial agents in animals indicated repeatedly the lack of information on the environmental fate of antibiotics (10). The paucity of information is related both to the lack of methodology and to the lack of the necessity for measuring stability and mobility of antibiotics in soils. Methodologies used in published studies were based on buffer extraction, desorption reactions, solvent extractions, and AOAC extraction procedures for feeds (11).

Table 1. Recovery of bacitracin from soils

Added, U/g	Found, U/g	Recovery, %
Adelphia Sandy Loam		
0.0	ND[a]	—
1.0	0.75	75.0
2.0	1.05	52.5
3.0	1.52	50.8
5.0	2.80	56.0
7.0	3.75	53.0
		Av. 57.5
		SD 8.9
Suburban Garden Soil		
0.0	ND	—
1.0	0.62	62.0
2.0	0.90	45.0
3.0	1.29	43.0
5.0	2.80	56.0
7.0	3.25	46.4
		Av. 50.5
		SD 7.3
Agricultural Sandy Soil		
0.0	ND	—
1.0	0.80	80.0
2.0	1.35	67.5
3.0	2.08	69.2
5.0	3.25	65.0
7.0	5.87	83.9
		Av. 73.1
		SD 7.4

[a] None detected.

Overall, these assay systems had limited sensitivity and could not measure low levels. Analytical procedures capable of measuring 1 μg/g soil or less are necessary to study the fate and effects of antibiotics in soils.

The soils used were obtained from agricultural areas and none had animal wastes applied to them for periods exceeding 3 years prior to samples being taken. The soils used contained a reasonable range of sand, clay, and silt.

The soils were mixed well but there was little or no certainty that the subsamples taken were homogeneous. Hence, replicate analyses of samples containing the same concentration were not performed on different days. Recoveries over the range of concentrations used, 1–10 μg or units antibiotic/g, and the standard deviations should give the analysts reasonable perspective as to expected sensitivities and recoveries.

The recoveries of bacitracin from the 3 soils used in these studies showed no distinct pattern related to either the clay, silt, or sand content, although the recovery of bacitracin from the agricultural sandy soil was slightly higher than experienced with the other test soils (Table 1).

Table 2. Recovery of tetracyclines from soils

Chlortetracycline			Oxytetracycline	
Added, μg/g	Found, μg/g	Recovery, %	Found, μg/g	Recovery, %
Adelphia Sandy Loam				
0.0	ND[a]	—	ND	—
1.0	0.40	40.0	0.42	42.0
2.5	1.50	60.0	0.77	30.8
5.0	2.45	49.0	1.75	35.0
10.0	9.70	97.0	4.00	40.0
		Av. 61.5		37.0
		SD 12.5		4.7
Suburban Garden Soil				
0.0	ND	—	ND	—
1.0	0.64	64.0	0.44	44.0
2.5	1.70	68.0	1.75	70.0
5.0	3.90	78.0	4.15	83.0
10.0	11.60	116.0	9.12	91.2
		Av. 81.5		72.1
		SD 20.6		17.9
Agricultural Sandy Soil				
0.0	ND	—	ND	—
1.0	0.57	57.0	0.83	83.0
2.5	2.22	88.8	2.50	100.0
5.0	4.40	88.0	3.50	70.0
10.0	8.50	85.0	8.50	85.0
		Av. 79.7		84.5
		SD 13.2		10.6

[a] None detected.

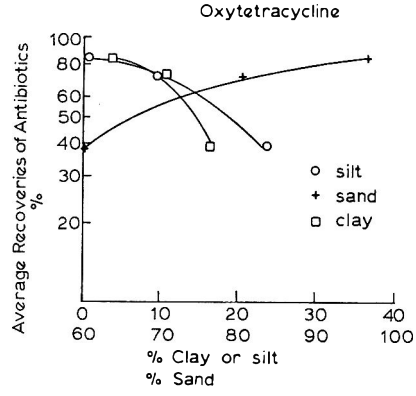

Figure 1. Recoveries of oxytetracycline from soils as a function of clay, sand, and silt content.

The recoveries were reasonably consistent within each soil. In the sandy loam, recoveries averaged 57.5% (50.8–75.0), in the garden soil 50.5% (43.0–62.0), and in the sandy soil 73.1% (65.0–83.9). The recoveries from the sandy soil were surprising because of the 96.3% sand content. If adsorption on clay or silt were a major factor, the average recoveries should have been lower in garden soil and lowest in sandy loam. Recoveries were unrelated to crude clay content and cannot be related to any specific type such as kaolinite or montmorillonite. A complete understanding of the reasons for the low recoveries is beyond the scope of the manuscript, although it should be noted that recoveries of bacitracin from animal feeds are also less than quantitative.

The solvent system methanol–pH 6.5 buffer (50 + 50) was superior to 100% methanol, methanol–water, or dimethylformamide extraction. This system permitted direct dilution with pH 6.5 buffer to recommended levels. No interferences from soils could be seen. The lower limit of measurement was below the intended 1 unit/g. The buffer system for the response line was modified to reflect any contribution of the solvent on the assay organism.

Acid–methanol was used as the extracting solvent for both chlortetracycline and oxytetracycline. The use of acid–acetone–water for the extraction of chlortetracycline yielded recoveries of 13–20% of added chlortetracycline. The recoveries of chlortetracycline at different concentrations in both the Adelphia sandy loam and the garden soil were variable, implying that the soil samples used were not totally homogeneous. There was no apparent relationship between the recoveries obtained and clay, silt, or sand content of the soil, although the greatest consistency of recoveries occurred in the agricultural sandy soil (Table 2).

In contrast, the recoveries of oxytetracycline appeared to be related to soil composition. Recoveries of oxytetracycline from Adelphia sandy loam averaged 37.0% (30.8–42.0%), in the garden soil 72.1% (44.0–91.2), and in the agricultural sand 84.5% (70.0–100.0%). As both the silt and clay content decreased, recoveries increased. In the garden soil, recoveries increased linearly with increasing concentration; in the other soils, recoveries were relatively constant (Figure 1).

The ability to extract and subsequently measure tylosin in soils is related to the composition of the soil being analyzed. Table 3 shows the wide differences in recoveries obtained in the 3 soils used. Only 6.5% of the tylosin could be recovered from Adelphia sandy loam; 37.4% (34.6–39.2%) from the garden soil; and 74.1% (66.0–82.2%) from the sandy soil. Recoveries of tylosin over a range of concentrations were reasonably consistent in the individual soils.

There was an inverse relationship between the recovery of tylosin and silt content and a direct relationship between sand content and recoveries. Although the relationship is not linear,

Table 3. Recovery of tylosin from soils

Added, μg/g	Found, μg/g	Recovery, %
Adelphia Sandy Loam		
0.0	ND[a]	—
1.0	ND	—
2.5	ND	—
5.0	0.30	6.0
10.0	0.69	6.9
		Av. 6.5
Suburban Garden Soil		
0.0	ND	—
1.0	ND	—
2.5	0.96	39.2
5.0	1.92	38.3
10.0	3.46	34.6
		Av. 37.4
		SD 2.0
Agricultural Sandy Soil		
0.0	ND	—
1.0	0.66	66.0
2.5	2.06	82.2
5.0	3.69	73.7
10.0	7.45	74.5
		Av. 74.1
		SD 5.7

[a] None detected.

there is an inverse relationship between clay content and recovery of tylosin (Figure 2).

Extraction of tylosin from soils by using solvents was no more successful than extractions

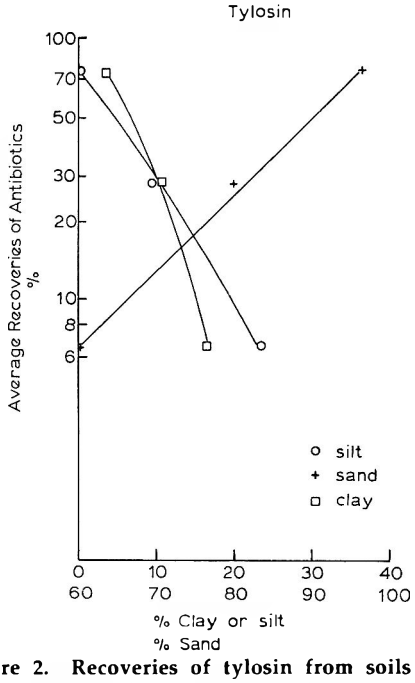

Figure 2. Recoveries of tylosin from soils as a function of clay, sand, and silt content.

Table 4. Recovery of erythromycin from soils

Added, μg/g	Found, μg/g	Recovery, %
Adelphia Sandy Loam		
0	ND[a]	—
1	0.33	33.0
2	0.63	31.7
3	0.86	28.7
5	1.50	30.0
7	3.10	44.3
10	5.00	50.0
		Av. 36.3
		SD 7.8
Suburban Garden Soil		
0	ND	—
1	0.40	40.0
2	0.93	46.7
3	1.50	50.0
5	2.54	50.7
7	4.00	57.1
10	6.67	66.7
		Av. 51.9
		SD 8.4
Agricultural Sandy Soil		
0	ND	—
1	0.83	83.0
2	1.87	93.3
3	2.17	72.2
5	3.67	73.3
7	5.45	73.8
10	6.03	60.3
		Av. 76.0
		SD 10.2

[a] None detected.

using pH 8.0 buffer at 60°C. The use of the methylal–methanol mixture (4 + 1), methanol, or methylal alone not only did not improve recoveries but resulted in the appearance of large zones of inhibition from blank soils which yielded large apparent levels of tylosin. The buffer extraction also did not yield blank values from unsupplemented soils; instead, small but measurable zones of inhibition occurred which calculated as 0.5 to 1.0 μg tylosin/g soil. The relatively poor extractability of tylosin would seem to indicate a relatively low potential for leach from soils, excepting those soils which have low clay and silt content. The stated need for a very sensitive assay system was not fulfilled in the case of tylosin, regardless of the extraction system used.

To ascertain if the relationship noted for tylosin was characteristic of the macrolide family, erythromycin was studied in the same soils. Recoveries of erythromycin were also related to soil characteristics. The best recoveries (Table

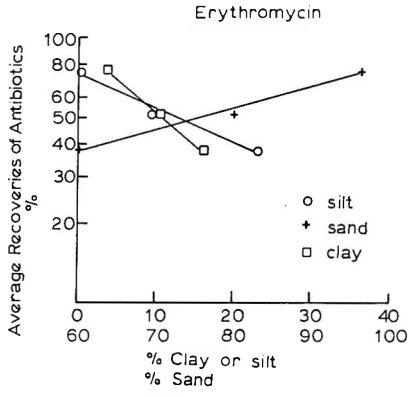

Figure 3. Recoveries of erythromycin from soils as a function of clay, sand, and silt content.

4) were obtained in sandy soil and the poorest in sandy loam. There was an inverse relationship between recoveries of erythromycin to both clay and silt content and a direct relationship with sand content (Figure 3). Both erythromycin and tylosin exhibited similar properties in the soils used, although actual recovery levels differed (Figures 2 and 3).

Recoveries of streptomycin from the soils used, regardless of clay, silt, or sand content, were zero. Streptomycin binds irreversibly to clays. Even in a soil of very low clay content, no added streptomycin could be measured.

Penicillin recoveries also were zero, reflecting the inherent instability of the molecule. Penicillin added to the soil was so unstable that within minutes after the addition of penicillin no activity could be extracted and measured. A water extract of the soil, filtered to remove soil particulates, degraded penicillin within minutes. Degradation was extremely rapid for all test soils and their extracts.

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PESTICIDE RESIDUES

Determination of Xanthates, Dithiocarbamates, and Some Fungicides by Titration with Electrogenerated Iodine in Anhydrous Acetonitrile

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Xanthates, dithiocarbamates, and the fungicides maneb and zineb were determined by titration with electrogenerated iodine in anhydrous acetonitrile. The electrolyte was potassium iodide. The generating electrode was a platinum spiral (2.5 cm diameter, 2 mm cross section). The enclosed cell compartment was continuously flushed with dry nitrogen, and sample solutions (0.20-0.40 mL) were introduced by syringe. With the aid of 2 platinum electrodes, the end point was indicated amperometrically. Ten minutes was required for each determination. The compounds were determined at ≥5 μequiv. with relative errors of 0.21-9%. Precision is 0.60-5.7%. Thiourea, sulfur, and urea did not interfere, but thioacetamide did. Solutions of maneb and zineb in dimethyl sulfoxide are stable enough that μequiv. amounts of each compound can be determined with a relative error of 7% or less.

It is not surprising that many methods have been developed for the determination of xanthates and dithiocarbamates because these compounds have been known for some time and have been used in industry, medicine, and agriculture (1, 2). For instance, the zinc and manganese salts of ethylenebis(dithiocarbamic) acid are the fungicides zineb and maneb, respectively.

The determinations of xanthates and dithiocarbamates, including a standard method of analysis for dithiocarbamate residues on crops (3), have been for the most part based on analysis of either carbon disulfide or the amine product from acid decomposition of the salt. Methods for the measurement of carbon disulfide have used iodometry (4, 5), gravimetry (6, 7), kinetics (8), gas chromatography (9–11), various types of spectroscopy (12–24), and phosphorescence (25). Likewise, gas chromatography (26), spectrophotofluorimetry (27), and titrimetry (28) have been used to determine the liberated amine from acid decomposition of dithiocarbamates.

Analytical methods designed to determine xanthates or dithiocarbamates by a process other than acid decomposition of the salts include polarography (29–32), chromatographic techniques followed by spectroscopic workup (33–37), and iodometry (38–42). Because many dithiocarbamates are not soluble in water, the application of these methods, in particular iodometry, has become more general by the use of anhydrous solvents (38–40). Verma and Kumar titrated dithiocarbamates with 0.05N iodine monobromide in acetonitrile (38). Paul et al. performed potentiometric titrations with iodine cyanide in alcohol, alcohol–chloroform, and acetone (39). Grand and Tamres titrated dithiocarbamates with iodine in chloroform, using visual and spectrophotometric methods (40). Maneb and zineb have not been mentioned in any of the studies.

Except for Karl Fischer titrations and the determination of aldehydes in 95% ethanol (43), no methods appear to have been reported for the coulometric generation of iodine in nonaqueous solvents.

The purpose of this paper is to report results for the determination of xanthates and dithiocarbamates, especially maneb and zineb, by the electrogeneration of iodine titrant in anhydrous acetonitrile.

Experimental

Apparatus

Titrations were performed in a cell (Leeds and Northrup Model 7961) with a polyethylene cover which snapped in place over the titration cell. The cover supported 2 platinum indicator electrodes, a septum through which samples were introduced by syringe, an inlet tube for the continuous introduction of dry nitrogen, a spiral generator electrode (2.5 cm diameter, 2 mm cross section), and a sintered-glass tube which contained and separated the cathode from the electrolyte in the main part of the cell.

A coulometric analyzer (Leeds and Northrup Model 7960) was used to generate iodine in the cell. An ampmeter was placed in series in the

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circuit so that any sudden change in the current efficiency could be noticed.

A Leeds and Northrup pH meter (Model 7401) was modified and connected to the 2 platinum indicator electrodes to observe the end point biamperometrically.

A Hamilton (WI798) syringe calibrated in 0.01 mL units was used to introduce samples into the titration cell.

Reagents

All solvents were reagent grade and were used without further purification. The commercial formulations for zineb and maneb were manufactured by Dexol Industries. The active ingredients in the maneb and zineb were labeled 80.0% and 75.0%, respectively.

Potassium xanthates and sodium dialkyl dithiocarbamates were prepared and purified by recrystallization from ethanol by procedures according to Klöpping and van der Kerk (44). The manganese and zinc ethylenebisdithiocarbamate were prepared by a procedure patterned after one by Aagrunol (45). The compounds were air-dried, stored in a desiccator, and used within 24 h after preparation.

Preparation of Disodium Ethylenebisdithiocarbamate (I)

Add 3 g ethylenediamine, 7.6 g carbon disulfide, and 50 mL water to 250 mL, three-neck, round-bottom flask fitted with separatory funnel and inlet tube for dry nitrogen. Stir mixture throughout reaction. Flush reaction flask with dry nitrogen. Immerse reaction flask in ice bath to keep temperature in the range 5–20°C during reaction. Slowly and dropwise add solution of 4 g NaOH in 40 mL water. Stir contents of flask 2 h. Evaporate to small volume. Filter and dry precipitate. Dissolve precipitate in absolute alcohol, and reprecipitate by adding absolute ether to mixture.

Prepare all other xanthates and alkyl dithiocarbamates in similar manner.

Preparation of Maneb and Zineb

Repeat above procedure for the preparation of I from ethylenediamine, carbon disulfide, and NaOH. To flask with I, add dropwise with constant stirring a solution of 0.05 mol MCl_2 (M = Mn or Zn) dissolved in 50 mL deionized water. Stir 2.5–3.0 h at 5–20°C. Filter resultant precipitate. Wash with deionized water and with absolute ethyl alcohol. Dry 24 h. Procedure is patterned from one designed by Aagrunol (45).

Chemical Analyses of Prepared Maneb and Zineb

The yellow manganese salt of ethylenebis(dithiocarbamic) acid decomposed at 134°C (lit. value 131°C (46)). The salt contained 19.1% Mn as determined by EDTA back-titration (47) (calcd 21.6%). Molecular weight by nonaqueous iodometry was 275.5 (calcd 265).

The white zinc salt of ethylenebis(dithiocarbamic) acid decomposed around 149°C. The salt was determined to contain 22.6% Zn by EDTA titration (46) (calcd 23.5%). A Perkin-Elmer Model 283 infrared spectrophotometer was used to record the spectrum of a KBr disc of the salt. The infrared spectrum exhibited strong bands at 3190, 1500, 960, and 370 cm^{-1}, confirming NH, thioureide, CS, and ZnS linkages, respectively. This agreed with that reported in the literature for the salt (48). Molecular weight by nonaqueous iodometry was 289 (calcd 275).

Solutions were prepared by weighing each compound in a 10 mL glass-stopper volumetric flask. Weights were recorded to the nearest 0.01 mg on a Sartorius balance. Xanthates were dissolved in absolute methanol or ethanol. Sodium alkyldithiocarbamates were initially dissolved in absolute alcohol and diluted with acetonitrile to the calibration mark of the volumetric flask. Maneb and zineb were dissolved in dimethyl sulfoxide (DMSO). All solutions were titrated immediately after each solution was completely dissolved.

The electrolyte solution was prepared by diluting saturated KI in acetonitrile with an equal volume of acetonitrile.

The solution for the cathode compartment was a mixture of glacial acetic acid and saturated KI in acetonitrile (3 + 7).

Preparation of Cell and Determination

Add 10 mL solution prepared from glacial acetic acid–saturated KI in acetonitrile (3 + 7) to cathode compartment. Pour 50 mL electrolyte solution into cell. Flush cell with dry nitrogen before and during each titration. Turn on magnetic stirrer and set dial to same speed throughout each titration. Turn on coulometer and generate just enough iodine to cause deflection of needle on pH meter. Adjust iodine content in cell to mark midway in full deflection on pH scale. Record position of midway mark and always return to this mark to achieve end point for each titration.

Introduce aliquots of each sample solution into cell by syringe. Set coulometric analyzer at load

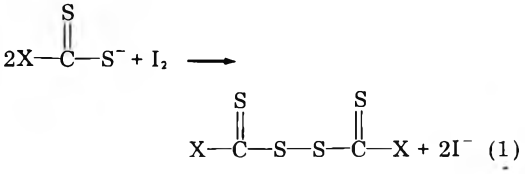
Table 1. Decomposition of dithiocarbamates dissolved in DMSO

Compound	Initial concn, M	Decrease in initial concn ½ h after dissolution	No. of measurements taken, ½ h
Maneb	0.0189	13.7%	8
	0.00867	11.1%	11
Zineb and sodium diethylcarbamate	0.01	no change (2 h)	

current of 6.43 mA. Begin titration and turn coulometer off just before needle on pH meter reaches pre-determined mark. Read μequiv. directly from coulometer.

Results and Discussion

Xanthates and dithiocarbamates are easily oxidized by iodine to dixanthogen and dithiuram disulfide derivatives, respectively.



$$2X-\overset{\overset{S}{\|}}{C}-S^- + I_2 \longrightarrow X-\overset{\overset{S}{\|}}{C}-S-S-\overset{\overset{S}{\|}}{C}-X + 2I^- \quad (1)$$

X = RO (xanthates)
X = RR'N (dithiocarbamates)

Grand and Tamres (40), Verma and Kumar (38), and Paul et al. (39) confirmed that the reaction occurs rapidly and completely in acetonitrile, alcohol, chloroform, and acetone. For the study reported in this paper, acetonitrile was chosen as a solvent because it has a high dielectric constant for an efficient electrochemical system, is resistant to oxidations and reductions, and readily dissolves potassium iodide, the electrolyte. Glacial acetic acid is added with acetonitrile to the cathode compartment of the coulometric cell to prevent the formation of a black precipitate at the electrode surface.

Because Keppel (3) reported that dithiocarbamates dissolve and decompose in DMSO, preliminary experiments were conducted to ascertain the degree of their stability in the solvent. The results are given in Table 1. Maneb was observed to decompose with time but not so fast that an accurate measurement could not be made of its concentration if samples were analyzed immediately after the salt dissolved. The concentration of solutions of zineb and sodium diethyldithiocarbamate did not change over a 2 h period. This observation agrees with that of Engst and Schnaak who reported that maneb decomposed more rapidly than zineb when air was bubbled through aqueous suspensions of the compounds (31).

Table 2. Titration of xanthates and dithiocarbamates

Compound	Taken, μequiv.	Found, μequiv.[a]	Rel. error, %
Xanthates (K salts):			
1-butyl	11.86	11.53 ± 0.33	2.8
3-methyl-1-butyl	7.00	7.28 ± 0.33	4.0
1-methyl-1-propyl	4.45	4.78 ± 0.26	6.0
1-propyl	6.56	6.55 ± 0.098	0.12
2-propyl	5.52	5.42 ± 0.12	1.8
Dithiocarbamates (Na salts):			
ethyl	37.7	34.7 ± 0.73	8.5
diethyl	12.87	13.35 ± 0.21	3.7
	25.13	23.93[b]	4.8
	51.72	50.75[b]	1.9
	78.54	78.10[b]	0.6
diisopropyl	26.66	24.1 ± 0.72	9.4
(1-methyl-1-propyl)	20.55	20.5 ± 0.12	0.1
Maneb	18.18	18.78 ± 0.11	3.3
	9.02	8.67 ± 0.39	3.9
Zineb	9.56	8.70 ± 0.32	9.0

[a] Mean ± SD for 10 determinations.
[b] Measurement based on single weighing of a solid sample.

The results for the determination of xanthates and dithiocarbamates are listed in Table 2. The coulometer was operated at a load current of 6.34 mA for these measurements. The data show that the procedure is accurate and reproducible for a variety of compounds with the dithio linkage. Ten trials were done for each compound. The sample size for a single trial ranged from 5 to 25 μequiv. Each deviation reported in Table 2 is the result of 10 trials of different sample sizes of each compound; it is the standard deviation over all sizes, i.e., it does not represent the standard deviation of 10 trials of 5 μequiv. of sample only. The overall standard deviations for all compounds ranged from 0.11 to 0.73. The relative error between added and found amounts was never greater than 10% and averaged around 4.0%. Experiments were also done with the coulometer set at a load current of 0.635 mA to measure the sensitivity limit of the method. Under these conditions, approximately 50 μg maneb could be determined with a relative standard deviation and error of 7% or less. The sensitivity limit is similar for the other compounds listed. The molar concentration of a 5.0 mL sample of 2×10^{-3}M solution of a potassium xanthate can be determined accurately within 5% error. This result for xanthate can be compared with that of Prasad et al., who titrated 2.6×10^{-4} to 1.1×10^{-3}M aqueous solutions of potassium salts of xanthates with iodine (42). They re-

Table 3. Comparison of titrimetric methods for determination of sodium diethyl dithiocarbamate

Titrant	Solvent	End point detection	Sensitivity, μg/mL	Sample size, mg	Concn	Rel. SD,[a] %
Electrogenerated iodine[b]	water	potentiometric and biamperometric	1.5	—	—	—
0.1N $AgNO_3$[c]	water	amperometric	—	15	10^{-3}M	0.2
0.05N IBr[d]	acetonitrile	visual and potentiometric	—	10	—	0.6
This work	acetonitrile	biamperometric	—	1.5	10^{-2}–10^{-3}M	2.0
	acetonitrile	biamperometric	40 (40 ppm)	0.04[e]	10^{-3}M	7.0

[a] Relative standard deviation is used as defined on p. 538 of *Chemical Analysis*, 2nd Ed. (1975), H. A. Laitinen and W. E. Harris, McGraw-Hill, Inc., New York, NY. RSD "is the standard deviation expressed as a fraction or percentage of the arithmetic mean. It is used mainly to show whether the relative or the absolute spread of values is constant as the values are changed."

[b] See Ref. 41.

[c] See Ref. 49.

[d] See Ref. 38.

[e] Maneb in DMSO.

ported a relative error of 0.1–1.5%. The end point was determined biamperometrically.

The results for the measurements of dithiocarbamates by the procedure in this paper are compared with other titrimetric methods in Table 3.

Gas chromatographic methods for residue studies of field samples have sensitivities of 0.56 to 7 ppm. In these studies, nickel electron capture (11), flame photometry (24), and other detectors were used to measure carbon disulfide from acid decomposition of dithiocarbamates. Although the procedure described in this paper was not tested for residues in the field, the sensitivity limit for pure samples was 40 ppm and could perhaps be improved with calibrated syringes capable of accurate delivery of smaller samples.

The coulometric procedure was used to analyze commercial blends of zineb or maneb dissolved in DMSO. When titrations were performed directly on these samples, the number of equivalents of iodine was larger than the theoretical amount by almost a factor of 20% or more. The author has no explanation for the large equivalent reading for the commercial blends because decomposition products or filler ingredients in the formulations were not known. It was shown that urea, sulfur, and thiourea do not interfere, whereas thioacetamide does. This indicates that the method is nonspecific for the formulations studied. However, the procedure was modified to monitor the metal dithiocarbamate content of commercial fungicides. The author carefully weighed a fixed amount of the original maneb formulation and treated the solid with repeated portions of chloroform and/or carbon tetrachloride. The purified residue was dried and weighed. The weight of the residue was 78.2% of the original (80.0% was reported on the label). The purity of the residue was quickly checked by the procedure cited above. The purity of dithiocarbamate was 98.0%.

In conclusion, this procedure is simple, rapid, and accurate. It does not require the preparation of calibration curves or the use of special reagents. It may have application for residue studies if interferences are carefully checked. Even so, it does not have the advantage of some methods such as mass spectrometry coupled with effective liquid chromatography, which is able to identify carbamic acid derivatives in amounts less than 1 ng (50). Nevertheless, the procedure has an advantage over time-consuming acid decomposition methods and is less expensive than some instrumental methods. This method is convenient as a rapid procedure for monitoring the purity and quality of dithiocarbamates.

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Determination of Trifluralin, Diallate, Triallate, Atrazine, Barban, Diclofop-Methyl, and Benzoylprop-Ethyl in Natural Waters at Parts per Trillion Levels

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A sensitive multiresidue method is presented for the determination of 7 neutral herbicides in natural waters, with practical detection limits between 5 and 100 ng/L (ppt). The extraction was carried out at pH ≤1, using dichloromethane, so that acid-type herbicides can also be incorporated in the future. The entire cleanup procedure involves base partitioning, preliminary cleanup and fractionation on a 10% deactivated Florisil column, and final cleanup of the fractions on an activated Florisil column. However, the cleanup procedure is flexible enough to permit the analyst to use some or all of the cleanup steps, depending on the herbicide levels of interest. Recoveries of the 7 herbicides from distilled and natural water samples were between 82 and 104% at 2 fortification levels. Furthermore, this basic analytical scheme has been demonstrated, separately, to be effective in the determination of other neutral compounds such as PCBs, 18 organochlorine pesticides, and chlorobenzenes and thus has a potential to be developed into a multiclass, multiresidue method.

As part of water surveillance and monitoring programs, there is a need to determine the more commonly used herbicides in water and in sediment. While there are some published methods for gas-liquid chromatographic determination of individual herbicides such as trifluralin (1), diallate (2), triallate (3), atrazine (4), and barban (5) in various substrates and a few reports (6, 7) on several herbicides in soil, there is no sensitive multiresidue method for determining neutral herbicides in water, particularly the 7 herbicides (Table 1) of interest to the Western Water Quality Branch regional laboratories. Furthermore, considerable sampling and analysis time could be saved if the same sample can be extracted and analyzed for both acid-type (phenoxyalkanoic acids) and neutral-type herbicides (carbamates, ureas, and esters). Therefore, we investigated a multiclass, multiresidue method for both the acid- and neutral-type herbicides.

Presented in this paper are the results of the first phase of our investigation, namely, a multiresidue method for the determination of 7 neutral herbicides in natural waters. This method can be integrated later with the acid herbicides to generate a multiclass method, as mentioned above.

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METHOD

Apparatus

(a) *Gas chromatograph.*—Model 5710 A equipped with ^{63}Ni electron capture detector and Model 7672A automatic liquid sampler (Hewlett-Packard, Avondale, PA 19311). Operating conditions: temperatures (°C)—injection port 200, column 185, detector 300; argon-methane (95 + 5) carrier gas flow rate 30 mL/min.

(b) *GLC columns.*—1.8 m × 2 mm id coiled glass columns packed with 3% OV-1 on 100–120 mesh Gas-Chrom Q (column 1), 3% OV-225 on 80–100 mesh Chromosorb W HP (column 2), 1.5% OV-17 + 1.95% OV-210 on 100–120 mesh Gas-Chrom Q (column 3) (Chromatographic Specialties Ltd, Brockville, Ontario, Canada, K6V 5W1) and Ultrabond 20M, 80–100 mesh (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.*—500 × 20 mm id with coarse fritted disk and Teflon stopcock (Fisher Scientific Co., Ltd).

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. 8 or pass distilled water through Millipore Super-Q unit (Millipore Corp., Bedford, MA 01730).

(b) *Sodium sulfate.*—Anhydrous reagent grade (BDH Chemicals, Toronto, Ontario, Canada M8Z

Table 1. Common and chemical names of 7 neutral herbicides and their practical detection limits in natural waters

Common name	Chemical name	Detection limit,[a] ng/L
Trifluralin	α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine	5
Diallate	*S*-(2,3-dichloroallyl)-diisopropylthiocarbamate	100
Triallate	*S*-(2,3,3-trichloroallyl)-diisopropylthiocarbamate	10
Atrazine	2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine	100
Barban	4-chloro-2-butynyl-*m*-chlorocarbanilate	100
Diclofop-methyl	methyl 2-[4-(2',4'-dichlorophenoxy)phenoxy]propionate	50
Benzoylprop-ethyl	ethyl 2-(*N*-benzoyl-3,4-dichloroanilino)propionate	25

[a] Based on 1 L sample and volume of extract made up to 10 mL for GLC analysis.

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, and spiking solutions in acetone.

(g) *Natural water samples.*—All natural samples came from various sites of Battle River, Old Man River, and Red Deer River in Western Canada.

(h) *Fortification of water samples.*—Add 100 μL herbicide mixture solution in acetone at appropriate levels to 1 L water in a whiskey bottle, stir, and equilibrate 30 min before extraction.

Extraction

Stir water sample (ca 1 L) collected in 40 oz long-neck whiskey bottle, using stirring bar covered with Teflon so that vortex formed at surface almost reaches bottom of bottle. Carefully add sulfuric acid (1 + 1) dropwise until pH is ≤1 (pH paper).

Add 50 mL dichloromethane and tightly cover bottle with piece of Teflon tape and cap. After stirring 30 min, transfer contents to 2 L separatory funnel. Transfer organic layer to 500 mL separatory funnel and transfer aqueous layer back to original sample bottle. Repeat the above procedure twice with two 50 mL portions of dichloromethane. Discard the aqueous layer after the last extraction. Shake combined organic extracts 1 min in 500 mL separatory funnel with 100 mL 2% $KHCO_3$ solution. Vent funnel periodically. After layers separate, check upper (aqueous) layer to make sure it is basic (pH paper).

Drain organic layer through sintered glass funnel containing 80 g (5 cm) anhydrous sodium sulfate and collect filtrate in 500 mL round-bottom flask.

Add 50 mL dichloromethane to 500 mL separatory funnel and shake 1 min. Pass organic layer through same Na_2SO_4 column as above. Rinse column with ca 50 mL dichloromethane. Collect filtrate and rinsings in same 500 mL flask described above.

Add 3 mL isooctane to combined dichloromethane extract in 500 mL flask. Evaporate extract on rotary evaporator to ca 10 mL under reduced pressure (water bath temperature ≤40°C). Add 50 mL hexane or petroleum ether and carefully evaporate to 3–5 mL as described above.

Florisil Column Cleanup

Prepare cleanup column by filling chromatographic tube with 20 g 10% deactivated Florisil. Tap column gently to settle adsorbent. Add 1 cm anhydrous Na_2SO_4 on top of Florisil layer.

Prewet column with 100 mL hexane and let solvent drain just to top of Na_2SO_4 layer. Discard hexane eluant.

Quantitatively transfer concentrated extract plus rinsings onto column with Pasteur pipet. When extract just enters Na_2SO_4 layer, elute column with 200 mL 25% benzene in hexane (Fraction A) followed by 200 mL 1% methanol in benzene (Fraction B). Collect each eluate in separate 500 mL round-bottom flask. Add 3 mL isooctane to each fraction before evaporation to 3 mL as described previously.

Minicolumn Cleanup

Prepare minicolumn by plugging 23 cm × 5 mm id 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 on top.

Prewet minicolumn with 2 mL hexane. After hexane has drained just to top of column, quantitatively transfer concentrated extract from Fraction A onto column with four 1 mL hexane rinsings. Elute column with additional 6 mL hexane. Discard hexane fraction.

Elute same column with 0.5% acetone in hexane and collect 10 mL eluate in graduated centrifuge tube (Fraction I; contains trifluralin, diallate, and triallate). To remove sulfur, shake this fraction with mercury on a Vortex-Genie mixer until metal is shiny. Analyze this fraction by ECD-GLC.

Prepare another minicolumn with activated Florisil and Na_2SO_4 as described above. Prewet minicolumn with 2 mL 25% benzene in hexane. After solvent has drained just to top of column, quantitatively transfer concentrated extract of Fraction B onto column with three 1 mL rinsings of same solvent mixture. Wash column with 7 mL of same solvent mixture. Discard this washing.

Elute column with 1.5% acetone in benzene. Collect 10 mL eluate in centrifuge tube (Fraction II, contains atrazine, barban, diclofop-methyl, and benzoylprop-ethyl). Analyze this fraction by ECD-GLC. (See also discussions on atrazine analysis.)

Results and Discussion

The analytical scheme is summarized in Figure 1. The experimental design of this investigation is based on a modular approach in method development in the sense that the developed method can be further expanded to other classes of compounds in the future. Addition of acid to the water sample before extraction is designed to incorporate a multiresidue procedure for acid herbicides (9, 10) or other acidic compounds at a later date. If only the neutral compounds (PCBs, organochlorines, and the 7 neutral herbicides) are of interest, adding acid before extraction can be omitted. For the 7 neutral herbicides, we have found that the efficiency of the extraction procedure is not pH-dependent. Furthermore, at the levels studied, no observable degradation of these 7 neutral herbicides occurred when samples were acidified to pH ≤ 1 before extraction.

Choice of Columns

Resolution of the 7 herbicides on 6 commonly used columns for pesticide analysis was investigated. In addition to the 4 columns mentioned in the apparatus section, the other columns investigated were 3% OV-17 and 4% OV-101 + 6% OV-210, both on 100–120 mesh Chromosorb W HP. The best resolution and response were obtained from a 3% OV-1 column, and the precision and recovery data were generated from it. A typical chromatogram of the 7 herbicide standards chromatographed on the OV-1 column is shown in Figure 2. For confirmation purpose, an Ultrabond 20M column is recommended because of reversal of elution orders of some compounds compared with the 3% OV-1 column (see Table 2).

Table 2. Retention times (min) of 7 neutral herbicides on different GLC columns [a]

Herbicide	Column 1	Column 2	Column 3	Column 4
Trifluralin	1.51	1.10	1.51	0.80
Diallate	1.70	1.26	1.58	0.97
Triallate	2.49	1.42	2.18	1.10
Atrazine	1.81	2.52	2.18	3.00
Barban	7.93	5.51	11.09	40.03
Diclofop-methyl	16.60	11.81	19.65	17.67
Benzoylprop-ethyl	19.61	18.43	26.15	19.03

[a] Column 1: 3% OV-1, 185°C, flow rate ca 30 mL/min; column 2: 3% OV-225, 245°C, flow rate ca 25 mL/min; column 3: 1.5% OV-17 + 1.95% OV-210, 200°C, flow rate ca 25 mL/min; column 4: Ultrabond 20M, 200°C, flow rate ca 25 mL/min.

For maximum GLC response of barban, an *N*-phenyl carbamate, some priming of the column is beneficial. It is, therefore, necessary to condition the columns by injecting a series of barban standards at a concentration a few times higher than those expected for analysis, until the response stabilizes. However, such treatment was not needed in the case of the OV-1 column.

The GLC response to barban may decrease gradually after the column is used over a period of time for sample analysis. If this occurs, replacement of the silanized glass wool at the column inlet will restore the signal response and reduce tailing.

During our investigation, it was observed that the GLC response to benzoylprop-ethyl, an amide, in pure hexane or isooctane solutions tended to be low and erratic. However, addition of 0.5% (v/v) acetone to these solutions increased and stabilized the response. This is similar to the situation for Kepone, as we and others (11) have observed.

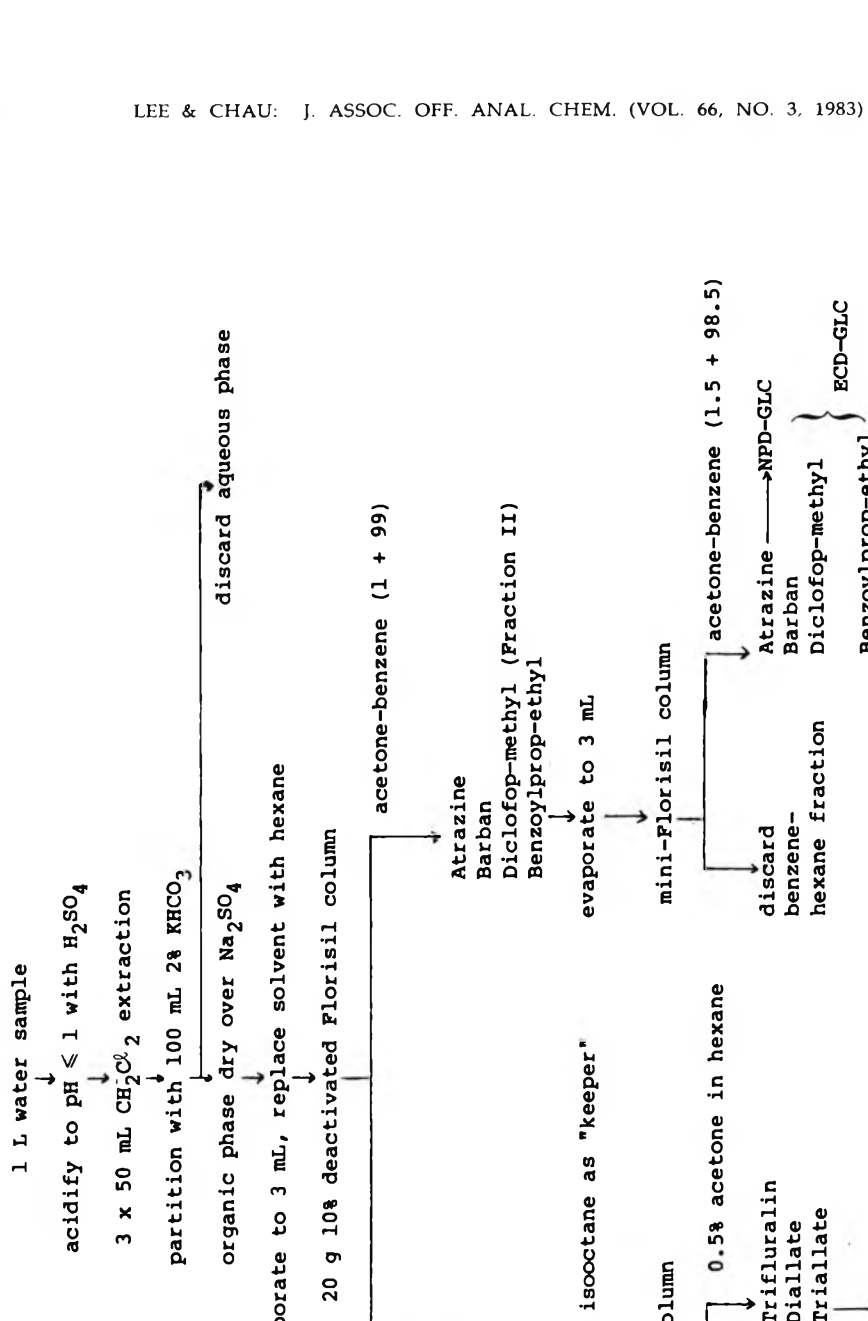

Figure 1. Analytical scheme.

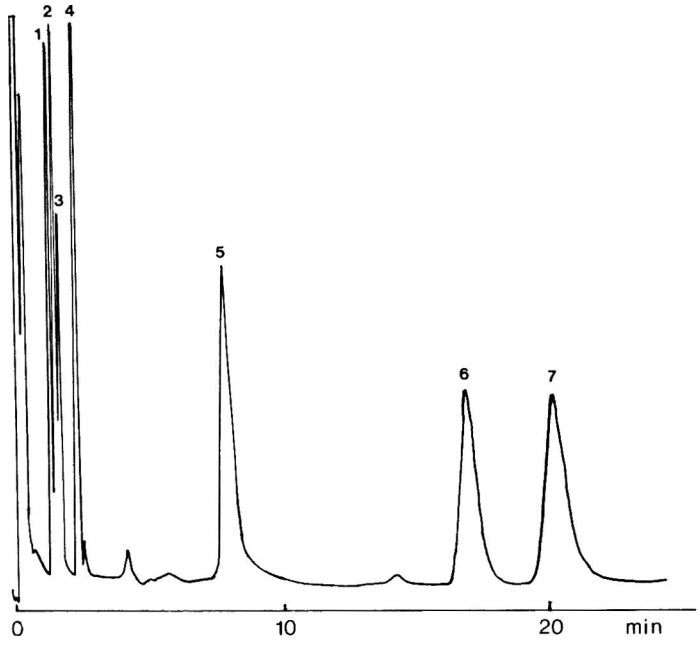

Figure 2. Gas chromatogram (ECD) of 7 neutral herbicide standards on 3% OV-1 column at 185°C: 1, 50 pg trifluralin; 2, 1 ng diallate; 3, 2 ng atrazine; 4, 110 pg triallate; 5, 1 ng barban; 6, 500 pg diclofop-methyl; 7, 250 pg benzoylprop-ethyl. Attenuation ×16.

Extraction Solvents

Dichloromethane was chosen as the extraction solvent for the 7 herbicides because this solvent has been shown to quantitatively recover PCBs (Aroclors 1242, 1254, and 1260) and 18 organochlorines, including mirex and photomirex, from natural water (12; unpublished results). This solvent was also demonstrated elsewhere to be a suitable extractant for at least 8 acid herbicides (10), several chlorobenzenes (12), and many organophosphorus pesticides (13) from water. Indeed, dichloromethane was used in a multiresidue method (14) for the extraction of several classes of organic compounds including the above from water. Thus, this solvent is suitable for the generation of a multiclass, multiresidue method.

PCBs, Organochlorines, and Chlorobenzenes

PCBs and some organochlorines are often encountered in environmental samples. Although not as frequent as PCBs, the occurrence of chlorobenzenes in samples is not uncommon. Because these compounds may pose interference problems in the determination of the 7 herbicides in question, elimination of their potential interference is desirable to increase the "ruggedness" of the current method. Also, it is our intention to eventually develop a somewhat comprehensive multiclass and multiresidue method for determining a variety of environmental pollutants. Therefore, these 3 classes of compounds were investigated briefly at the Florisil column fractionation stage.

As already mentioned, PCBs and 18 organochlorines can be quantitatively extracted at ppb and ppt levels by the present procedure. Whether one is interested in a multiclass extraction and analysis or not, it is essential to establish whether PCBs and these organochlorines can pose interference problems in the determination of the 7 neutral herbicides. We found in our preliminary investigations that all the PCBs, the less polar organochlorines, and the 12 chlorobenzenes are eluted quantitatively in the hexane fraction from the Florisil cleanup procedure. (Unlike for PCBs and organochlorines, the extraction efficiency of dichloromethane has not been investigated for all chlorobenzenes from natural water.) The more polar organochlorines that are coeluted in the 0.5% acetone in hexane fraction (Fraction I) are much less volatile (longer retention time) and will not interfere with the GLC analysis of trifluralin, diallate, and triallate.

Table 3. Single operator precision and % recovery (mean ± SD) for analysis of 7 neutral herbicides in fortified distilled water and natural waters

Herbicide	Level, μg/L	Distilled water (n = 6)	Natural water (n = 9)
Trifluralin	0.05	93.5 ± 3.5	97.9 ± 4.9
	0.005	—	94.1 ± 6.1
Diallate	1.0	91.3 ± 2.7	92.2 ± 1.3
	0.1	—	101.2 ± 8.5
Triallate	0.11	96.1 ± 1.9	96.5 ± 2.6
	0.011	—	103.9 ± 4.6
Atrazine	2.0	91.0 ± 3.2	95.2 ± 2.5
	0.2	—	89.7 ± 2.8
Barban	1.0	95.0 ± 5.6	99.3 ± 4.9
	0.1	—	99.3 ± 8.7[a]
Diclofop-methyl	0.5	97.8 ± 2.2	97.5 ± 2.8
	0.05	—	81.6 ± 5.3
Benzoylprop-ethyl	0.25	97.6 ± 2.5	103.0 ± 2.6
	0.025	—	89.5 ± 8.6

[a] n = 8.

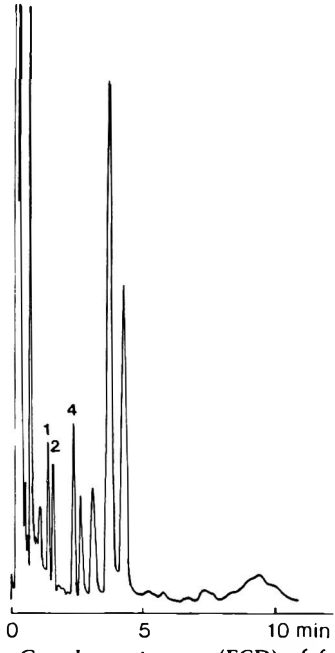

Figure 3. Gas chromatogram (ECD) of fraction 1 after cleanup from 1 L Battle River water spiked at or near detection limits: 1, 0.005 μg trifluralin/L; 2, 0.1 μg diallate/L; 4, 0.011 μg triallate/L.

Selection of Adsorbents for Column Chromatography

During the course of investigation in the selection of adsorbents for the column chromatographic cleanup of neutral herbicide extracts, various combinations of solvent systems, types of adsorbent, and amounts of deactivation were examined. Most of the work was done on the 3 commonly used adsorbents, namely, alumina, Florisil, and silica gel. Alumina, either activated or deactivated with 2 and 5% water, was shown to be undesirable for diclofop-methyl, because recoveries of this herbicide from alumina columns were low (≤80%). Thus, the use of alumina was ruled out for the initial column cleanup of sample extracts, although the adsorbent was still useful for further cleanup of fractions which did not contain diclofop-methyl. Even though all 7 herbicides were quantitatively recovered from silica gel columns, this adsorbent was less effective for removal of sample coextractives. Attempts had been made to chromatograph the herbicides on activated Florisil columns; however, varying activities of activated Florisil from different lots often caused overlapping of herbicide fractions, thus making reproducible elution patterns difficult to obtain. The adsorbent of choice was 10% deactivated Florisil because it gave quantitative recoveries (≥95%) of all 7 compounds, reproducible elution fractions, and satisfactory cleanup.

It should be pointed out that although the column elution pattern of the herbicides has been demonstrated on 3 different lots of Florisil of different densities from 2 suppliers (Fisher Scientific Co. and Supelco, Inc.), variations in adsorbent activities may still be found from batch to batch. The analyst is, therefore, urged to check the herbicide elution pattern with his or her own Florisil and standard solutions.

Selection of Cleanup Procedure

The cleanup scheme described above was designed and validated to provide adequate sample cleanup for the detection of 7 herbicides in water samples down to the detection limits. However, for the analysis of herbicides at higher levels or in cleaner samples, it was not necessary to go through the entire procedure, to save time. Our work indicated that only the initial base partitioning cleanup is required for the removal of interferences in water samples with herbicide levels at 100 times the detection limits or higher. For most screening applications, the base partitioning together with the 10% deactivated Florisil column cleanup are adequate for samples with herbicide levels at 10 times the detection limits or higher.

Precision, Recovery, and Detection Limits

The single operator precision and percent recovery from distilled water and natural waters are given in Table 3. The lowest levels investi-

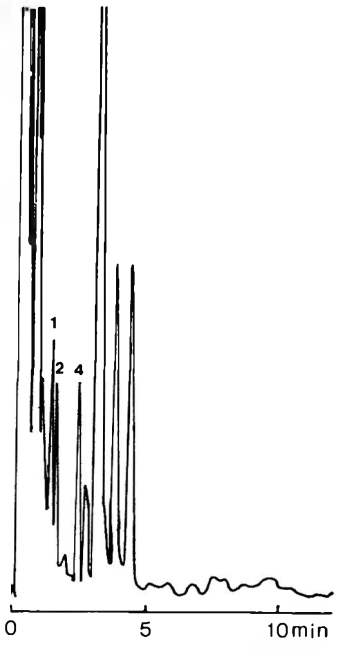

Figure 4. Gas chromatogram (ECD) of fraction 1 after cleanup from 1 L Old Man River water spiked at or near detection limits. For peak identification and concentrations, see Figure 3.

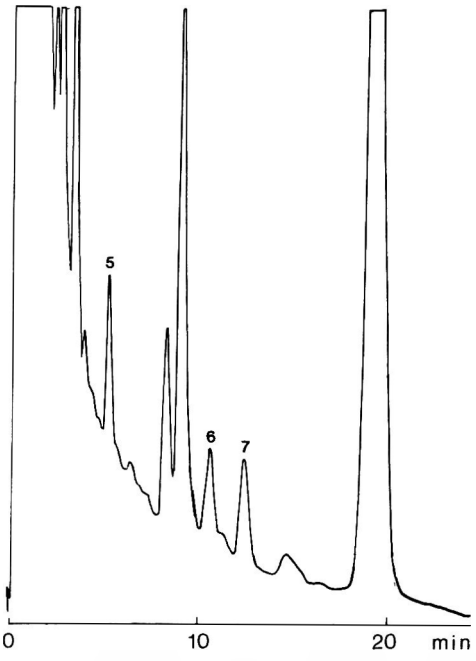

Figure 6. Gas chromatogram (ECD) of fraction 2 after cleanup from 1 L Battle River water spiked at or near detection limits: 5, 0.1 μg barban/L; 6, 0.05 μg diclofop-methyl/L; 7, 0.025 μg benzoylprop-ethyl/L. Chromatogram was run at 200°C. Note that atrazine in this fraction was detected only by NPD.

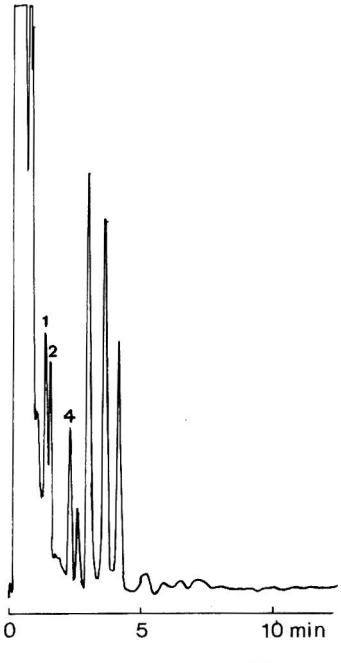

Figure 5. Gas chromatogram (ECD) of fraction 1 after cleanup from 1 L Red Deer River water spiked at or near detection limits. For peak identification and concentrations, see Figure 3.

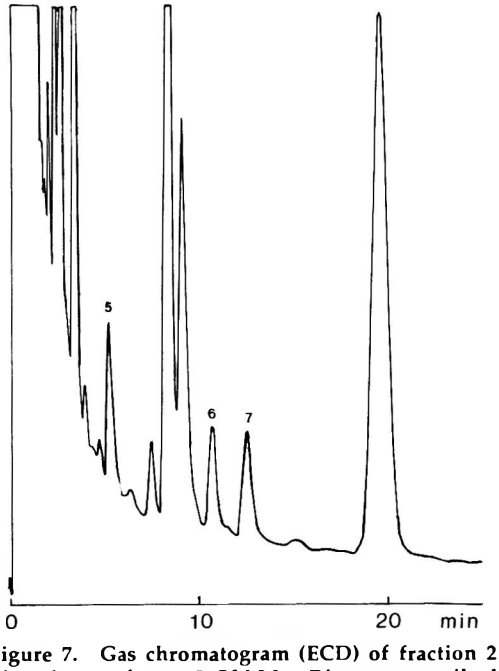

Figure 7. Gas chromatogram (ECD) of fraction 2 after cleanup from 1 L Old Man River water spiked at or near detection limits. For peak identification and concentrations, see Figure 6.

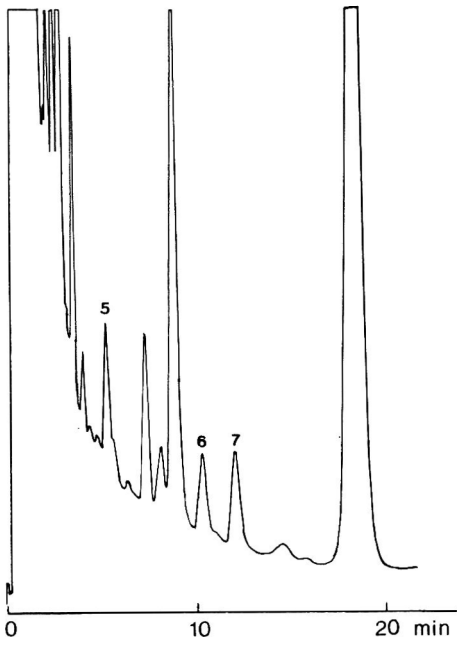

Figure 8. Gas chromatogram (ECD) of fraction 2 after cleanup from 1 L Red Deer River water spiked at or near detection limits. For peak identification and concentration, see Figure 6.

gated are the practical detection limits for the natural waters investigated. Four types of water were used in this investigation, namely, distilled water and water from 3 locations in western Canada. These natural waters contained various amounts of suspended solid and humic substances ranging from light yellow to brownish in color. Detection limits are known to vary with water quality and coextractives present; therefore, for different waters, one can generate higher or lower detection limits than our reported values by adjusting the volume of extract for GLC analysis.

It should be noted that the low detection limits for trifluralin (0.005 ppb) and triallate (0.01 ppb) cannot be obtained for the other compounds because the absolute responses of those herbicides to ECD (in the case of atrazine with the NPD, see discussions below) are some 10 to 20 times lower than responses of trifluralin or triallate. Representative chromatograms of extracts of water samples spiked at the detection limits after all necessary cleanup are shown in Figures 3–8.

Atrazine Analysis

In the above method, ECD-GLC was used for the analysis of all 7 herbicides. However, a specific detector such as the nitrogen/phosphorus detector (NPD) (Model 18789A, Hewlett-Packard) gave better specificity and sensitivity than ECD for atrazine. Using this detector, the practical detection limit can be lowered to 0.1 μg/L.

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Determination of 2-Chloroethanol in Honey, Beeswax, and Pollen

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Procedures were developed and tested for the determination of residues of 2-chloroethanol (ethylene chlorohydrin, ECH) in honey, beeswax, and pollen. Recoveries of ECH from fortified samples averaged 91, 87, and 89%, respectively, for each substrate. The maximum amount of ECH found in substrate fumigated with ethylene oxide was 36 μg/g in honey, 124 μg/g in beeswax, and 132 μg/g in pollen. A tendency was noted for darker waxes, which contain larger amounts of naturally occurring chlorides than light-colored waxes, to contain the greater amounts of ECH. A gas-liquid chromatograph equipped with a Dohrmann halogen-specific detector was used for identification and quantitation.

Ethylene oxide (ETO) is a broad spectrum antimicrobial gas which has received increasing attention during the last 2 decades for the control of American foulbrood disease (transmitted by spores of *Bacillus larvae*) in the larvae of the honeybee (*Apis mellifera*) (1, 2). Compared with the traditional burning of diseased colonies and hives to destroy the causative organism, sterilization of the hives with ETO offers the promise of avoiding the substantial replacement cost of the hive.

However, Wesley et al. (3) reported finding ethylene chlorohydrin (2-chloroethanol, ECH) in spices known to contain inorganic chlorides and which had been fumigated with ETO. ETO itself was not found in the samples examined by these authors. Apiary products can be expected to contain naturally occurring inorganic halides, and beeswax taken from frames fumigated with ETO and sealed and frozen from 1 to 4 days after fumigation contained measurable levels of ECH and no ETO (H. Krehm & R. Scharfe, Agriculture Canada, private communication).

The LD_{50} of ECH has been reported between 64 and 98 mg/kg, depending on the test species (4), placing it in the "very toxic" category (5). It is absorbed readily by ingestion (6), and there is some evidence that it is also mutagenic (7).

As a result of its known toxicity and the strong possibility that this water-soluble compound might migrate from the wax or be formed in the moisture-containing honey, an analytical procedure for the determination of ECH in honey was developed. Because pollen present in the frame at the time of fumigation may also contain ECH, following fumigation, and because it may be moved about the hive during the feeding of the young, several methods of extracting ECH from pollen were examined. Recovery data for extraction and quantitation of ECH from beeswax are also presented.

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METHOD

Apparatus

(a) *Chromatographic tubes.*—22 mm od × 650 mm, with Teflon plug and coarse fritted disk.

(b) *Centrifuge tubes.*—15 mL capacity, graduated, conical with Teflon-lined screw caps.

(c) *Gas chromatograph.*—MicroTek Model GC.2000MF equipped with Dohrmann microcoulometric detector operated in the halogen-specific mode. Operating conditions: injector 250°C, column 124°C for both columns, transfer line 260°C, detector furnace 820°C.

(d) *Columns.*—2.4 m × 6 mm od borosilicate glass, injector port length packed with silanized glass wool, balance packed with: (i) 20% Carbowax 20M-TPA on 80–100 mesh Gas-Chrom Q; (ii) 15% Reoplex 400 (polypropylene glycol adipate) on 80–100 mesh Supelcoport.

(e) *Blender.*—Polytron (Brinkmann Instruments, 50 Galaxy Blvd. Rexdale, Ontario, Canada).

Reagents

(a) *Standards.*—2-Chloroethanol (anhydrous), purity not stated (J.T. Baker Chemical Co., Phillipsburg, NJ 08865); *p*-dichlorobenzene, 99% (International Scientifica Ltd, 3028 Laguna St, San Francisco, CA 94123). Prepare individual solutions in ethyl ether containing 2.5, 5.0, 7.5, and 10×10^{-7} g 2-chloroethanol/mL and 10^{-6} g *p*-dichlorobenzene/mL.

(b) *Solverts.*—Acetone, ethyl ether, and petroleum ether; distilled in all-glass systems.

(c) *Xylene.*—Reagent grade (BDH Chemicals).

(d) *Paraffin solution.*—1% paraffin oil in heptane.

(e) *Florisil.*—60–100 mesh, 5% w/w deactivation; prepare according to Currie (8).

(f) *Sodium sulfate.*—Anhydrous, coarse, granular. Heat 48 h at 600°C to drive off impurities.

Cool, and store in glass containers with Teflon-lined caps.

(g) *Water.*—Petroleum ether-extracted or other interference-free water.

(h) *Cheesecloth.*—Washed twice in enough ethyl ether to cover cloth.

Extraction

Honey.—If honey is in frames, scrape ca 100 g honey and comb from foundation and place on cheesecloth secured over 400 mL beaker. Place in oven at 60°C and collect filtered honey (60–70 g). Weigh 50 g filtered honey (or other extracted honey) directly into 500 mL separatory funnel. Add 50 mL warm water (ca 50°C) to funnel, stopper, and shake to dissolve honey. Let cool to ambient temperature, add 100 mL ethyl ether, stopper funnel, and shake contents vigorously 1 min, venting as necessary. Let layers separate ca 20 min, at which time obtain (from top) organic layer, interfacial layer (emulsion), and aqueous layer. Drain aqueous layer into 250 mL beaker and retain. Collect interfacial layer into 250 mL Erlenmeyer flask containing ca 60 g Na_2SO_4, swirl until emulsion breaks, and let stand. Drain organic layer remaining in separatory funnel through 50 g Na_2SO_4, collecting it in 250 mL evaporator flask. Then decant organic layer in Erlenmeyer flask through same Na_2SO_4 filter. Return aqueous layer to separatory funnel, add 100 mL ethyl ether, mix vigorously 1 min, and let layers separate ca 20 min. Discard aqueous layer. Collect interfacial and organic layers as described above, including breaking of emulsion and drying through Na_2SO_4 filter. Add 0.5 mL paraffin solution to contents of evaporator flask and reduce solvent volume to ca 12 mL on rotary evaporator at 30°C. Transfer contents of evaporator flask to 15 mL graduated centrifuge tube using 2 mL rinse of ethyl ether.

Beeswax.—The method reported by Brown (9) for ETO and ECH in plastic and rubber surgical equipment was modified for beeswax and is similar to that used in ref. 4.

Weigh 5 g wax into 400 mL beaker, add 100 mL xylene, and macerate 1 min, using Polytron blender. Heat wax–xylene macerate to ca 50°C on explosion-proof hot plate (at 1/3 heat) in fume hood. Using powder funnel, place 25 g Florisil into chromatographic tube and tap with wooden rod or heavy cardboard tube to settle adsorbent. Place 10 g Na_2SO_4 on top of Florisil layer and tap tube to settle layers. Into 13 cm diameter filtering funnel, place Whatman No. 4 filter paper and 25 g Na_2SO_4. Place filter assembly on top of chromatographic tube and place 400 mL beaker under tube. Pour warm wax–xylene mixture into filter assembly and let filtrate drain directly onto column. Rinse beaker with 50 mL xylene and use rinse to rinse funnel after first portion of xylene has just completely passed into adsorbent column. When xylene meniscus has again reached top of Na_2SO_4 layer in chromatographic tube, elute column with 140 mL petroleum ether. Discard eluants. Place 250 mL evaporator flask under column and elute ECH from column with 100 mL ethyl ether. Proceed from "Add 0.5 mL paraffin solution . . ." as in procedure for honey.

Pollen—Method 1.—Weigh 5 g pollen into glass mortar containing 25 g Florisil and grind together with pestle to form free-flowing mixture. Transfer sample–Florisil mixture into extraction thimble. Place thimble in Soxhlet extractor fitted with 250 mL boiling flask containing 180 mL ethyl ether and a few boiling chips. Adjust reflux rate to obtain 3–4 cycles/hr. Extract for 18 h, cool, and proceed from "Add 0.5 mL paraffin solution . . ." as in procedure for honey.

Pollen—Method 2.—Mix 5 g pollen with 15 g Florisil as described in Method 1. Using powder funnel, add 15 g Florisil to chromatographic tube, followed by pollen–Florisil mix and 10 g Na_2SO_4. Tap chromatography tube to settle contents after each addition of material to tube. Place 250 mL evaporator flask under column and elute with 175 mL ethyl ether. Place second 250 mL evaporator flask under column and elute with 100 mL ethyl ether. For each flask proceed from "Add 0.5 mL paraffin solution . . ." as in procedure for honey.

Pollen—Method 3.—Proceed as in Method 2, substituting ethyl ether–acetone (4 + 1 v/v) for ethyl ether.

Detection and Quantitation

Inject 30 μL (equivalent to ca 10 or 100 mg) sample onto GLC column (i). Quantitatively measure by comparing peak heights to those obtained by injecting same volume of standard. If response from sample exceeds full scale deflection of strip-chart recorder, dilute sample extract so that response is on scale. Detector response to ECH was linear in the range of 10–40 ng injected on column, provided that intercept of +4 ng was used in calculation. Confirm identity of ECH using *p*-value (10) and/or GLC column (ii).

Using described GLC conditions, 15 ng ECH injected onto column gave detector response of 40% full-scale. Based on detector response of 6%

Table 1. Recovery of 2-chloroethanol from fortified honey, beeswax, and pollen [a]

Substrate	Fortification range, μg/g	Av. rec., %	SD
Honey [b]	0.05–2.0	91	6
Beeswax [b]	0.4–5.0	87	7
Pollen [c]	0.5–4.0	89	6

[a] Pollen by Method 1 only.
[b] Based on 13 determinations.
[c] Based on 9 determinations.

full-scale (ca 2 cm), injection of ca 11 mg of extracted pollen or wax results in lower limit of detection of 300 ng/g, while injection of ca 110 mg extracted honey results in a detection limit of 30 ng/g. Retention time on each column was ca 4.5 min. The relative retention time (vs *p*-dichlorobenzene) was 0.64 for column (i) (Carbowax 20M-TPA) and 0.72 on column (ii) (Reoplex 400). *p*-Value for ECH between ethyl ether–water (1 + 1) was 0.47.

Results and Discussion

For recovery studies, honey was fortified with ECH at the 0.05, 0.2, 0.4, and 2.0 μg/g levels, beeswax at the 0.4, 1.0, 2.0, and 5.0 μg/g levels, and pollen at the 0.5, 1.0, 2.0, and 4.0 μg/g levels. Samples were allowed to equilibrate for approximately 1 h after fortification and then analyzed by the described procedures (for pollen, only pollen method 1 was tested). The results are presented in Table 1. Recoveries of ECH from honey ranged from 77 to 98%, from beeswax from 77 to 97%, and from pollen from 81 to 98%. All substrates for the recovery tests had been pre-analyzed by the appropriate procedure and contained no detectable amounts of ECH. No biasing of recoveries with regard to fortification level was noted.

Addition of paraffin solution to the extract before the evaporation stage is essential to obtaining satisfactory recoveries. It is also important that the volume of solvent not be allowed to decrease below about 10 mL and in no case should the sample be allowed to evaporate to dryness.

Pollen known to contain ECH was collected from several frames and mixed thoroughly with a mortar and pestle. Duplicate subsamples of 2 g each were analyzed by Methods 1, 2, and 3 described for pollen. Results are given in Table 2. Not unexpectedly, the greatest amount of ECH was extracted by using the 18 h Soxhlet method. The lowest recovery was obtained with the ethyl ether–Florisil column technique, with an intermediate amount being extracted using the ethyl ether–acetone–Florisil system. The 2 frames from which this pollen was collected had been fumigated in March or April 1980, used in active hives for the 1980 season, removed from the hives in September, and stored outdoors until analyzed in January 1981. It is assumed that the pollen was present in the frame at the time of fumigation, but this was not documented.

Table 2. 2-Chloroethanol (μg/g) extracted from composite fumigated pollen sample by Methods 1, 2, and 3

Method	Sample	2-Chloroethanol Fraction 1	2-Chloroethanol Fraction 2
1	1	116	—[a]
	2	132	—[a]
2	1	65	9
	2	55	14
3	1	84	16
	2	72	21

[a] Not applicable.

As the analyses of samples progressed, it became apparent that darker waxes tended to contain the larger amounts of ECH, as did the honey contained in that wax. Dark honeycomb wax is an older wax containing more biological impurities than foundation wax and might be expected to contain greater amounts of chloride than the newer wax. Because naturally occurring chlorides have been associated with the production of ECH in other products (3), a chloride analysis was performed on 9 wax samples for which the ECH levels were known for both the wax and the honey. As can be seen in Table 3,

Table 3. Comparison of color, chloride content (μg/g), and 2-chloroethanol (ECH) content (μg/g) of beeswax and 2-chloroethanol content of honey (μg/g) from frames fumigated with ethylene oxide

Frame	Wax Color	Wax Chloride	Wax ECH	Honey, ECH
1	white	185	ND[a]	0.4
2	dark	641	33	—[b]
4	dark		55	15
5	dark	720[c]	97	14
6	dark		62	33
7	dark		80	31
8	dark	720	124	36
9	white	95	10	6
10	light	254	18	12

[a] Not detected (<300 ng/g).
[b] No honey available in this frame.
[c] Value of composite sample from frames 4, 5, 6, and 7.

Table 4. 2-Chloroethanol residues (μg/g) found in wax and honey taken from brood chambers fumigated in March 1980, used on active hives for summer, and analyzed January 1981

Frame	Wax	Honey
1-a	0.3	0.60
2-a	ND[a]	0.05
3-a	ND	0.13
4-a[b]	0.5, 0.8	0.65, 0.76
1	0.5	0.22
2	2.0	0.31
5	1.1	0.20

[a] Not detected (<300 ng/g).
[b] Analyzed in duplicate.

Table 5. 2-Chloroethanol residues (μg/g) in honey extracted from hives in which fumigated (see Table 4) brood chambers had been used for 1980 season

Sample	Amt found
1	0.09
2	0.11
3	0.09
4	0.22
5	0.12
6[a]	0.18

[a] Extracted approximately mid-season.

the darker waxes do contain the most chloride. No correlation can be noted between ECH levels in the wax and the honey.

Brood chambers, which had been fumigated with ETO in March 1980, were placed on active hives for the summer and collected for analysis in January 1981. The beeswax from these brood chambers contained ECH ranging from none detected (<300 ng/g) to 2 μg/g (7 frames), while the honey from these frames contained ECH ranging from 0.05 to 0.76 μg/g (Table 4).

Honey extracted from supers which were on the hives containing the fumigated brood chambers contained ECH ranging from 0.09 to 0.22 μg/g for 6 samples, one of which was extracted in mid-season (Table 5). The presence of ECH in honey away from the treated frames may be attributed to the known practice of bees moving honey from brood chambers to supers and vice versa (R. Topping, Alberta Agriculture, private communication, 1981).

A single sample of wax known to contain ECH was analyzed for ETO plus ECH by using the reaction column described in ref. 10. Aliquots of the sample analyzed with and without the reaction column gave values of 8.0 and 8.3 μg/g, respectively. These values are within the experimental error of the analytical method; therefore, one must deduce that no ETO was present in the sample. This result was in accordance with those reported in refs. 3 and 4.

Acknowledgment

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Rapid Method for Extraction and Reverse Phase Liquid Chromatographic Determination of Paraquat Residues in Water

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A simple and fast analytical method is described for the quantitative determination of low levels of paraquat residues in water. The method involves extraction and concentration of paraquat in water by using a C_{18} Sep-Pak cartridge followed by reverse phase high performance liquid chromatographic determination with ultraviolet detection at 257 nm. Recoveries of paraquat from spiked samples were above 93% with a coefficient of variation of 6.1%. The method can be used for water samples with paraquat concentrations as low as 0.05 ppm.

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dication) is a herbicide which is a very effective contact desiccant. It is widely used in the preharvest desiccation of various crops, for postemergent nonselective weed control, and for aquatic weed control (1). In addition to its use on commercial crops, paraquat has been used to destroy marijuana in Mexico by aerial spraying (2).

Trade names (3) for paraquat include Gramoxone, Preeglone, Weedol, and Aerial Gramoxone. Paraquat compounds are very soluble in water, and stable to acid solution. On controlled pyrolysis, paraquat loses its methyl groups to form 4,4-bipyridyl (4).

Analysis of paraquat in formulations (5), soil (6–8), plant tissues (9–14), and biological samples (15–31) have been reported. Methods for determining paraquat in water include bioassay (32, 33), thin layer chromatography (34, 35), colorimetry (36–38), and gas chromatography (4, 28, 39). These methods require extensive sample treatment, and are time consuming.

The purpose of the present investigation was to develop a simple analytical method that allows rapid analysis of low levels of paraquat residues in water.

METHOD

Apparatus and Reagents

(a) *High performance liquid chromatograph.*—Perkin-Elmer Series II with Rheodyne sample injector Model 7120 (with 100 μL loop) or injector Model 7150 equipped with Perkin-Elmer variable wavelength ultraviolet (UV) detector, Model LC-55, and Hewlett-Packard integrator Model 3380A. Chromatographic conditions: temperature, ambient; flow rate, 2 mL/min; wavelength, 257 nm; chart speed, 0.5 cm/min; and injection volume, 100 μL with 100 μL loop.

(b) *Chromatographic column.*—Whatman Partisil PXS 10/24 ODS stainless steel, 25 cm long × 4.6 mm id (Whatman Inc., Clifton, NJ 07014).

(c) *Guard column.*—Stainless steel, 7.0 cm long × 4.6 mm id, laboratory packed with Co:Pell ODS (Whatman Inc.).

(d) C_{18} Sep-Pak™ cartridge.—Waters Associates, Milford, MA.

(e) *MiniPump®.*—Milton Roy Co., St. Petersburg, FL.

(f) *Reagents.*—Paraquat dichloride (analytical grade 100% purity) was supplied by Quality Assurance Section, Analytical Chemistry Branch, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711. Analytical grade monobasic ammonium phosphate ($NH_4H_2PO_4$) (Mallinckrodt Chemical Works), reagent grade 85% phosphoric acid (American Scientific and Chemical), Gram-Pacs® borax ($Na_2B_4O_7 \cdot 10H_2O$) buffer, and pesticide grade acetonitrile (Fisher Scientific Company) were obtained from local suppliers. Organic-free water, used in this work, was prepared by passing distilled water through a Milli-Q™ water purification system (Millipore Co., Bedford, MA 01730).

Vermilion River water was collected from a location near St. Joseph's General Hospital, Vegreville, Alberta, Canada.

(g) *HPLC mobile phase for gradient elution.*—*Solution A:* Dissolve 23.01 g monobasic ammonium phosphate in 500 mL 30% acetonitrile–water, and adjust pH of resulting solution to 3.0 with phosphoric acid. *Solution B:* 30% acetonitrile–water. Gradient: 0–20% A at 9% A/min.

(h) *Eluant.*—Dissolve 2.88 g monobasic ammonium phosphate in water in 100 mL volumetric flask, add 1.0 mL phosphoric acid, and dilute to volume with water.

(i) *Stock solution.*—0.5 μg paraquat dichloride/μL. Accurately weigh 50 mg paraquat dichloride, dissolve in water in 100 mL volumetric flask, and dilute to volume with water.

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Table 1. Recovery of paraquat from spiked water samples [a]

Sample No.	Fortification [b] level, ppm	Vol. sample concentrated, mL	[Paraquat]$^{++}$ present, μg	[Paraquat]$^{++}$ recd, μg	Rec., %
1	1.00	50	36.18	33.72	93.2
2	0.75	100	54.28	52.54	96.8
3	0.50	100	36.18	34.45	95.2
4	0.25	100	18.09	19.68	108.8
5	0.10	100	7.24	7.60	105.0
6	0.05	200	7.23	7.09	98.0
$\bar{X}$					99.5
SD (n = 6)					6.1
CV, %					6.1

[a] Each value is an average of 2 determinations.
[b] Paraquat dichloride.

Preparation of Spiked Samples

Transfer 0, 50, 100, 250, 500, 750, and 1000 μL portions of stock solution with the appropriate syringe into separate clean 500 mL volumetric flasks. Dilute each to 500 mL with water. Concentrations of paraquat dichloride in above samples are 0.00, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00 ppm, respectively.

Determination

Dissolve 1.90 g borax buffer in 500 mL spiked sample and filter resulting solution through 0.45 μm MF-Millipore filter (HP WP 04700) with all-glass filtration apparatus (Millipore Co.).

Treat C_{18} Sep-Pak cartridge by passing 10 mL methanol through it, followed by 5 mL water at ca 3 mL/min. A glass syringe with Luer end fitting or other appropriate device may be used for this purpose.

Quantitatively pass 100 mL (or as required) of above filtrate, with MiniPump, through pretreated cartridge at 2.5 mL/min. Collect effluent from cartridge in 100 mL volumetric flask to confirm volume of water concentrated.

Wash cartridge with 10 mL water at 2.5 mL/min. Drain and discard water from cartridge, and elute paraquat with eluant solution at ca 1.0 mL/min. Collect first 5 mL eluate in 5 mL volumetric flask.

Make 3 replicate 100 μL injections of concentrated sample into liquid chromatograph and calculate amount of paraquat by external standard method.

Results and Discussion

The use of C_{18} Sep-Pak cartridges to purify paraquat concentrate extracted from marijuana has been described (9). In the present investigation we used such cartridges to extract and to concentrate dissolved paraquat from water. A procedure (40) previously used for determining difenzoquat in water was modified for use in this study. The analytical column was conditioned by passing HPLC mobile phase (20% A + 80% B) through it for about 2 h before use. For calculating recovery, the peak area obtained by injecting 100 μL of the concentrated sample was compared with the peak area obtained by injecting an equivalent amount of paraquat in 100 μL of the eluant solutions. Recoveries of paraquat from spiked water samples are reported in Table 1. Average recovery was 99.5% with a coefficient of variation of 6.1%.

Paraquat recovery decreased to <60% when potassium dihydrogen phosphate (1.70 g/500 mL) or sodium bicarbonate (0.50 g/500 mL) was used as an ion pair reagent in the concentration step. The same result was obtained when concentration of paraquat was attempted without the addition of ion pair reagent.

Some peak broadening occurred when the injection volume was increased from 2 μL (1 μg paraquat dichloride) to 100 μL (1 μg paraquat dichloride). The peak was much broader with a 175 μL injection. Peak broadening also took place when the concentration of solution A in the mobile phase was increased slowly (1–2% A/min).

Linearity of the UV detector response for paraquat was verified by injecting 100 μL of eluant solutions in which the amount of paraquat dichloride was varied from 100 ng to 2 μg. Results were plotted as peak areas vs amount of paraquat injected. The curve was linear over the concentration range examined (r = 0.998), and gave a lower limit of detection of 100 ng of the injected material (signal-to-noise ratio = 2).

To determine within-run precision, 5 water samples were spiked at 0.25 ppm paraquat di-

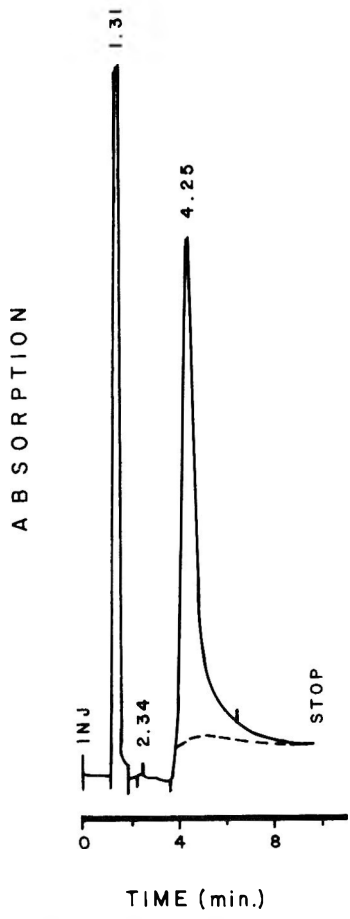

Figure 1. Chromatogram of 0 (---) and 1 ppm (—) paraquat dichloride in Vermilion River water.

chloride, and carried through the above procedure. Average recovery of paraquat from these samples was 92% with a coefficient of variation of 4.9%.

To demonstrate the use of this method, Vermilion River water was filtered through a 0.45 μm filter and spiked with paraquat dichloride at 1.0 ppm level in triplicate. Spiked samples as well as unspiked samples were carried through the above procedure. Results showed no paraquat in the blank samples; the average recovery of paraquat from the spiked samples was 93%. Figure 1 is a chromatogram of 0 and 1 ppm paraquat dichloride in Vermilion River water. The paraquat peak was at 4.25 min. Other peaks at 1.31 and 2.34 min were due to some unknown compounds present in Vermilion River water.

This method presents a simple, accurate, rapid, and quantitative procedure for determining paraquat in water. It has a potential for application to the analysis of other bipyridylium herbicide residues in water.

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CEREAL FOODS

Rate Nephelometric Measurement of Wheat Germ in Pasta Products

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An immunoassay for the rate nephelometric determination of wheat germ added to pasta products has been developed using a specific wheat germ antiserum. The method can be used to analyze germ from either soft or hard wheat. Specially prepared pasta products and commercial preparations were analyzed with the immunonephelometric method, in comparison with the radial immunodiffusion technique; good correlation was found between the results of the 2 assays.

There are 2 official methods in Italy for the immunological measurement of proteins in food: one for testing soft wheat and one for testing wheat germ in pasta products. The fact that the ingredients are listed on the packages of the products results in the need to control their presence or absence. Without a control method, the product cannot be traded in Italy. The official immunological method is based on radial immunodiffusion according to Mancini et al. (1) but we have used immunonephelometry to quantitate the antigen-antibody reaction.

In view of the results obtained in the immunonephelometry determination of soft wheat in pasta products (2), we have applied this technique to the quantitative determination of wheat germ. The kinetic nephelometric method is based on the measurement of maximum rate of change of light intensity scattered by an antigen-antibody complex and provides a quantitation of antigen. In this case the antigen is a specific protein of wheat germ that is normally present in very low concentration in pasta products prepared with hard wheat semolina. However, some manufacturers produce special pasta with added wheat germ to improve nutritional value. The immunonephelometric technique gives results in a few minutes (3) while the radial immunodiffusion method requires 24 h or more for complete analysis.

In this paper we describe the immunonephelometric determination of wheat germ and report the comparison of results obtained with a group of samples analyzed both with the proposed method and with the official method (4).

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Experimental

Apparatus and Materials

(a) *Nephelometer.*—Beckman immunochemistry system "ICS" (Beckman Instruments Inc.) equipped with dispenser, 42 μL automatic pipet, nephelometry buffer, reaction cuvets, magnetic stirrer, and optically encoded cards for manual operation. The instrument has been described in detail elsewhere (5).

(b) *Reference samples.*—Containing 1.5, 3, 6, and 10% (w/w) wheat germ (supplied by the Agnesi Pasta Factory, Imperia, Italy). Prepared after Cantagalli et al. (4) had demonstrated that widely used wheat germ from 3 different varieties (Iesi, Canadian Amber, and Maremma) had a specific protein content that was not significantly different.

(c) *Anti-wheat germ serum.*—Produced from rabbit, by ISVT Sclavo Siena, Italy, and directed against a specific protein of wheat germ with a molecular weight of about 40.000 present both in hard and in soft wheat germ (6, 7). The gamma globulin fraction was purified and stabilized for use in the nephelometric technique (8) and diluted to a protein concentration of 3.9 mg/mL.

(d) *Pasta samples.*—Ground in an electric grinder (Bühler, Italy); sample extracts were centrifuged using a Sorvall RC5 centrifuge.

Sample Extraction

Sample extracts were obtained as follows: 0.5 g ground pasta products were mixed in a test tube with 1 mL water containing 0.1% NaN_3, and after 30 min at room temperature, were centrifuged at 12 000 × *g* for 15 min. The supernate was transferred into a new test tube and centrifuged again.

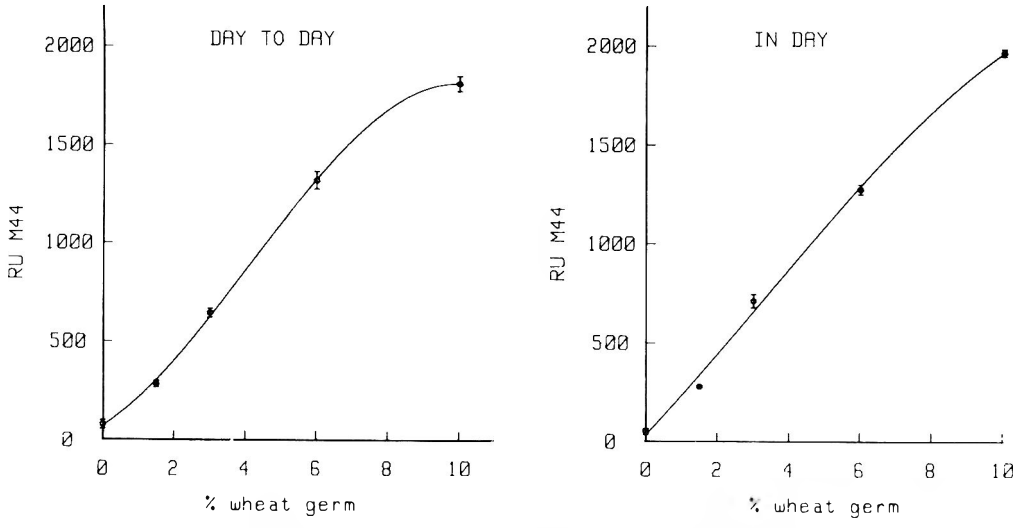

Figure 1. Day-to-day and within-day reference curves with immunonephelometric method. Each point is reported in figure as mean ± 2× standard error.

Immunonephelometric Analysis

The pasta extract was diluted 1:3 with water containing 0.1% NaN_3. The instrument was programmed with M CAL and M44 cards for manual operation. Reaction cuvet containing 600 μL magnetically stirred nephelometry buffer was inserted in the apparatus, 42 μL sample extract was added, and, when message "inject sample" appeared on display, 42 μL anti-wheat germ gamma globulin was added. When the "option" button was pressed, ca 60 s later, the value of the rate signal appeared on display in arbitrary rate units (RU).

The day-to-day reference curve and the within-day reference curve were plotted by repeating this operation for every reference pasta sample twice a day for 12 days (day-to-day experiments) and 12 times on the same day (within-day experiments). The mean concentration of wheat germ was calculated with standard deviation by reading on the respective curve the day-to-day values and the within-day value for every one of the 19 pasta samples.

Radial Immunodiffusion

Preparation of the slides and of the antiserum-agarose mixture, execution of the analysis, and dyeing were performed according to Cantagalli et al. (4). Day-to-day and within-day experiments were also performed with this method.

Results and Discussion

We have performed wheat germ determinations on 19 pasta samples produced by Italian factories. Some of these were special pasta products with added wheat germ produced by the Agnesi Pasta Factory. The results obtained

Table 1. Precision of rate nephelometric wheat germ test compared with radial immunodiffusion

Sample	*N*	Mean (% wheat germ)	SD
	Nephelometric Day-to-Day Precision		
High	24	8.1	0.47
Medium	24	4.3	0.40
Low	24	2.4	0.39
	Within-Day Precision		
High	12	8.6	0.40
Medium	12	4.5	0.32
Low	12	2.6	0.36
	Radial Immunodiffusion Day-to-Day Precision		
High	24	8.4	0.39
Medium	24	4.6	0.42
Low	24	2.5	0.41
	Within-Day Precision		
High	12	8.3	0.29
Medium	12	4.1	0.18
Low	12	2.6	0.28

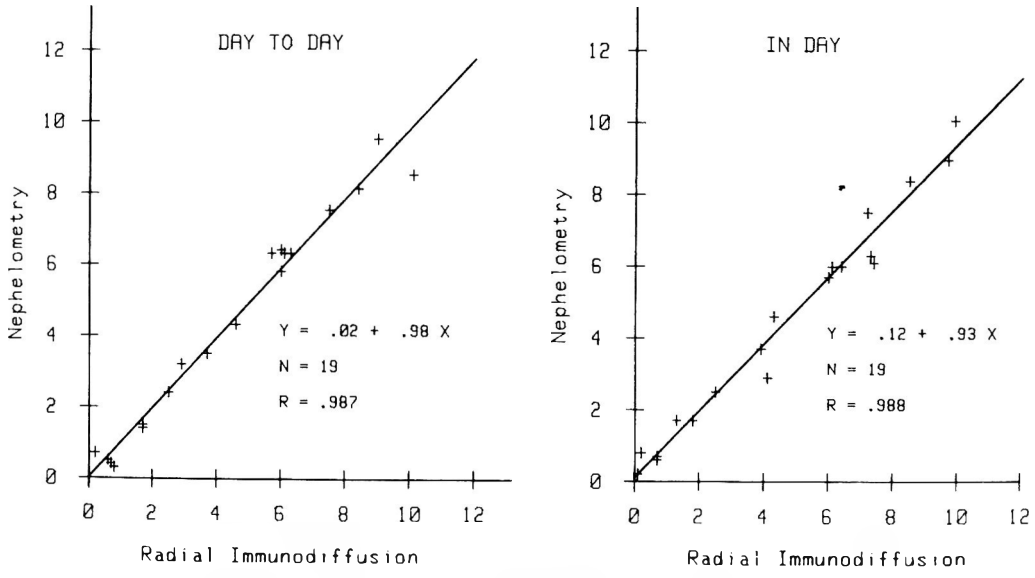

Figure 2. Comparison between results obtained with rate immunonephelometric wheat germ test and radial immunodiffusion test.

with the immunonephelometric method and with the radial immunodiffusion method were analyzed according to classical statistical methods (9) to show the correlation between them. The programs were prepared on a Hewlett-Packard 9845A-desk top computer equipped with a 9872 plotter for drawing graphs.

Figure 1 shows the day-to-day and within-day reference curves calculated with nonlinear regression, using a third degree polynomial expression to determine the relationship between dose and value of rate signals in the immunonephelometric method.

Table 1 shows day-to-day and within-day precision for both methods in samples with high, medium, and low amounts of wheat germ.

We set up the method with a measuring range varying between 0 and 10% (w/w) of added wheat germ. However, there is only a remote possibility that samples may contain more than 8% germ because high contents give the pasta a bad taste and poor storage characteristics. We thus neglected the antigen excess detection procedure suggested by Beckman.

In Figure 2, a comparison of the data obtained on the 19 samples with immunonephelometric and radial immunodiffusion methods shows that the 2 techniques correlate well. In fact, the linear regression lines have a high correlation coefficient for both day-to-day and within-day data (r = 0.987 and 0.988, respectively).

Moreover, the intercept values (0.02 for day-to-day and 0.12 for within-day) do not differ significantly from 0 ($P > 0.05$), and the regression coefficients (0.98 for the former, 0.93 for the latter) do not differ significantly from 1 ($P > 0.05$).

The immunonephelometric method allows the analysis of 20–25 unknown samples in 3–4 h, including the time required for weighings and preparation of sample extracts.

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COLOR ADDITIVES

High Pressure Liquid Chromatographic Determination of Tartrazine in Rice Milk Following Ion-Pair Extraction with Tri-*N*-Octylamine

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Tartrazine is separated from the dried and powdered rice milk matrix by elution with methanol-ammonia mixtures, the extract is purified by ion-pair extraction with tri-*n*-octylamine and back-extraction with sodium perchlorate, and the dye is determined by high pressure liquid chromatography.

In a previously published paper (1), we reviewed several techniques used in the quantitative analysis of food dyes. The applicability of one of these techniques, e.g., the ion-pair extraction with tri-*n*-octylamine (TnOA), was demonstrated by us in several papers (1-3). However, before the dyes can be extracted, they must be solubilized. It is the aim of this paper to describe the quantitative liberation of tartrazine (E102, FD&C Yellow No. 5) from rice milk, a rather complicated food matrix containing fats, proteins, and carbohydrates. Dyes are subsequently solubilized, extracted with TnOA, and identified and determined by ion-pair reverse phase high pressure liquid chromatography (HPLC) (1, 3, 4). The procedure is applied to the analysis of commercial samples.

Experimental

Apparatus and Reagents

(a) *Chromatograph.*—Varian LC 5020 equipped with manual Valco injector (loop size 100 μL); Varian 254/280 nm UV detector; 300 × 4 mm id column of MCH-10 (Micro-Pak 10 μm octadecylsilica); Varian CDS 111 integrator.

(b) *pH meter.*—Orion Ionalyser 601 and combined glass + calomel electrode.

(c) *Glass chromatography tubes.*—40 cm × 1 or 2 cm id, equipped with Teflon stopcock.

(d) *TLC plates.*—Cellulose precoated plates, 20 × 20 cm (Merck, GFR), with layer thickness of 0.1 mm.

(e) *TLC eluant.*—Ethyl acetate-*n*-propanol-ammonia-water (35 + 35 + 20 + 20).

(f) *Mobile phase.*—Prepare two 1 L solutions of methanol-phosphate buffer (pH = 7.00 ± 0.05, ionic strength 0.1): (a) 30 + 70 and (b) 60 + 40, each containing 0.5% (v/v) tetrabutylammonium hydroxide solution (TBA) (25% in methanol, Fluka AG). Mobile phase consists of 85% (a) + 15% (b).

(g) *Tartrazine.*—95% pure (P. Entrop, Machelen, Belgium), used as received. Purity degree was not considered in calculating % recovery or dye content of samples.

(h) *Tri-n-octylamine.*—(Aldrich Europe, Beerse, Belgium) used as received.

(i) *Buffers.*—With constant ionic strength (0.1), prepared as follows: pH = 5.5: 1.26 g $Na_2HPO_4.2H_2O$ and 24.65 g $NaH_2PO_4.H_2O$ dissolved in double-distilled water and diluted to 2 L. pH = 7.0: 9.38 g $Na_2HPO_4.2H_2O$ and 5.77 g $NaH_2PO_4.H_2O$ dissolved in double-distilled water and diluted to 2 L. If necessary, pH was adjusted to 5.50 ± 0.05 or 7.00 ± 0.05 with 0.1M sodium hydroxide.

Extraction

Two g rice milk, dried by lyophilization, was transferred to a glass chromatography tube of 1 or 2 cm id; the dyes were eluted with varying amounts of an elution mixture consisting of ammonia and methanol at an elution rate of ca 0.5 mL/min. The eluate was evaporated to dryness at ca 40°C, the residue was redissolved in 20 mL phosphate buffer (pH = 5.5, ionic strength 0.1), and the dye was extracted with 5 mL 0.1M TnOA solution in chloroform; 3 mL of the chloroform phase was re-extracted with 3 mL 0.1M sodium perchlorate.

Quantitative Determination

A calibration curve was constructed by plotting the area of the HPLC peak vs the concentration of the tartrazine standard. Over a concentration range from 0 to 50 mg/kg, a regression coefficient of 0.9996 was obtained. The amount of tartrazine in the extracts was determined by a curve-fitting program. All determinations were carried out in triplicate. The limit of detection was 5 ng (100 μL of a pure tartrazine solution, 0.05 mg/kg) at 0.01 AUFS.

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Table 1. Recovery of tartrazine in rice milk (using 1 and 2 cm columns) as a function of eluant volume (methanol–ammonia (95 + 5))

Eluant, mL	Rec., % 1 cm	Rec., % 2 cm
25	71.9	—
50	73.2	—
100	100.3	24.0
200	—	26.2
300	—	30.3

Table 2. Recovery of tartrazine from rice milk as a function of composition and volume of eluant (methanol–ammonia)

Eluant (% methanol)	Rec., % 25 mL	50 mL	100 mL
100	25.3 ± 1.4	58.6 ± 7.9	100.0 ± 3.4
95	71.9 ± 0.5	73.2 ± 0.2	100.3 ± 0.8
90	79.1 ± 1.6	93.7 ± 5.1	100.9 ± 1.6
85	67.0 ± 4.5	82.3 ± 4.1	86.0 ± 0.7
80	82.0 ± 2.0	89.9 ± 3.8	87.3 ± 1.8

Results and Discussion

Selection of Chromatography Tube

Two chromatography tubes with different internal diameters (1 and 2 cm) were used. Equal amounts (2 g) of dried rice milk were analyzed in each tube. The analytical rice milk was prepared from a tartrazine-free sample to which 50 mg/kg of tartrazine was added. The dye was eluted with amounts ranging from 25 to 300 mL of a methanol–ammonia mixture (95 + 5). This mixture was also used by Lehmann et al. (5) in the desorption of acid synthetic dyes adsorbed on polyamide. The results in Table 1 indicate a clear effect of the diameter of the chromatography tube. Because of the longer contact time of eluant with substrate in the 1 cm tube, a much higher recovery is obtained with small elution volumes (25–100 mL).

With the 1 cm tube, a 100% extraction yield was obtained with 100 mL eluant so it was not necessary to elute the rice milk with greater amounts of eluant. With the 2 cm tube, the amount of dye extracted with 25 and 50 mL eluant was negligible and was not even measured. Higher amounts of eluant did not give satisfactory recovery (ca 30%). In subsequent investigations, the tube with 1 cm id was used.

Influence of Eluant Composition and Volume

The eluant used by Lehmann et al. (5) to desorb dyes from polyamide consisted of a methanol–ammonia mixture; therefore, we investigated the influence of the composition of such an eluant on the extraction yield. At first, the recovery of tartrazine from rice milk was studied at a concentration of 50 mg/kg. The results listed in Table 2 indicate an obvious effect of the elution volume: At a concentration of 100, 95, and 90% methanol, 100% extraction yield is observed when 100 mL eluant is passed through the column. The influence of the volume of eluant is minimal at a concentration of 80% methanol and is maximal when 100% methanol is used. An increase in ammonia content increased recovery, especially with elution volumes of 25–50 mL. At methanol concentrations of 85 and 80%, the effect is not so pronounced: With volumes of 25 and 50 mL, the extraction yield goes through a minimum at 85% but when volumes of 100 mL are used, extraction yields are quite similar at 85 and 80% methanol. When amount of dye added to the rice milk is varied, one notes a slight decrease of extraction yield with increasing dye concentration (Table 3) at an eluant composition of 85% methanol. However, at 95% methanol, recovery is quantitative over the range of 10–100 mg/kg.

Analysis of Commercial Samples

At the request of the Food Inspection of the Belgian Ministry of Public Health, 13 different commercial rice milk samples were analyzed using 150 mL methanol–ammonia (95 + 5) eluant. Sample pretreatment consisted of lyophilization of the rice milk and grinding to powder in a mortar. This step was added to the method to obtain a homogeneous dye dispersion and therefore a representative sample. Tartrazine was detected in 3 samples; its identity was confirmed by its R_f value in a TLC system and by its retention time in HPLC. The amounts obtained are given in Table 4, the chromatograms (monitored at 0.08 AUFS) are given in Figure 1. Fol-

Table 3. Recovery of tartrazine from rice milk (elution volume 25–100 mL (85 + 15)) as a function of amount spiked

Amount, mg/kg	Rec., % 25 mL	50 mL	100 mL
10	66.7 ± 9.8	82.8 ± 3.5	88.4 ± 9.5
50	67.0 ± 4.6	82.3 ± 5.1	86.0 ± 0.7
100	73.5 ± 7.9	75.8 ± 2.5	77.8 ± 0.5

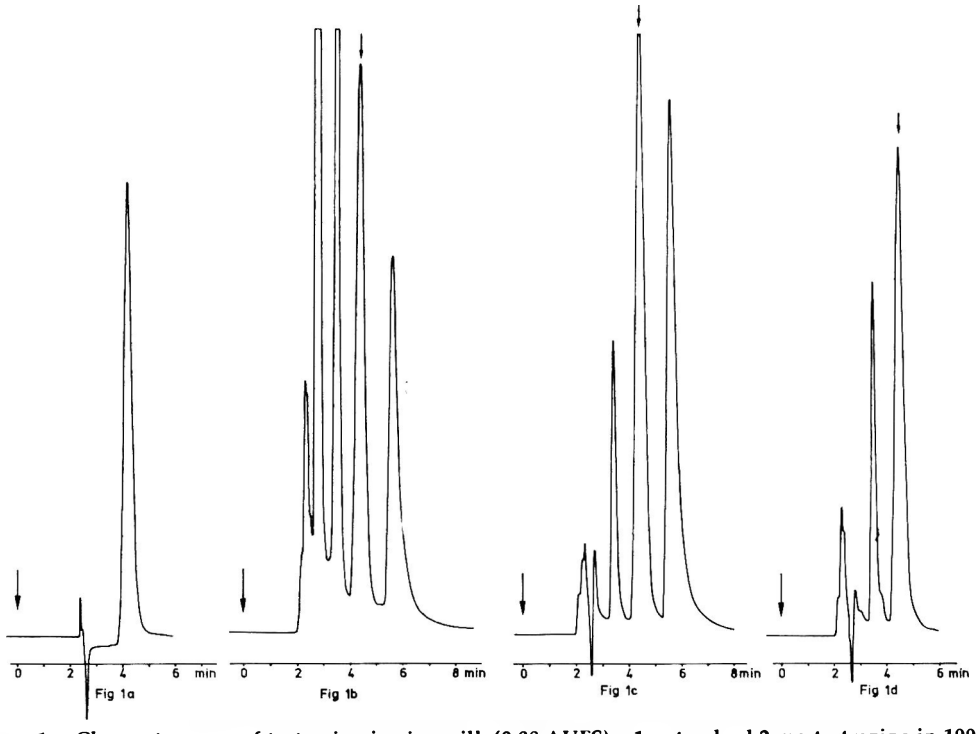

Figure 1. Chromatograms of tartrazine in rice milk (0.08 AUFS): 1a, standard 2 mg tartrazine in 100 mL double-distilled water. 1b, 1c, and 1d, Samples 1, 2, 3. Extract of sample 3 was diluted with equal volume of water before injection.

Table 4. Tartrazine content of commercial rice milk samples

Sample	Dry substance, mg/kg	Rice milk, mg/kg
1	43.4 ± 1.2	12.4 ± 0.4
2	70.0 ± 0.2	21.2 ± 0.1
3	78.8 ± 4.2	18.1 ± 1.0

lowing the extraction scheme given above, the detection limit is 0.2 mg/kg rice milk at 0.01 AUFS. The method is specific for tartrazine since the combination of TnOA extraction followed by the perchlorate back-extraction extracts only anionic dyes and, furthermore, tartrazine is resolved from other dyes by the HPLC system.

The method described here could also be applied to other foods and other dyes as long as the foods are solid and may be packed in a chromatography tube. However, it is important to note that the analyzed amount of sample should be adjusted in such a way that detection is possible and that the ion-pair extraction with TnOA yields a quantitative recovery. It is well known that in ion-pair extraction a sufficient excess of counter ion is required to obtain quantitative extraction. Therefore, if one wants (for example) to extract tartrazine from highly colored rice milk (>100 mg/kg), it would be preferable to decrease the amount of sample analyzed to 0.5 or 1 g.

Acknowledgments

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INDUSTRIAL CHEMICALS

Gas-Liquid Chromatographic Determination of *S,S,S*-Tributyl Phosphorotrithioate (DEF) in Water and Fish Tissue

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Methods are described for determination of *S,S,S*-tributylphosphorotrithioate (DEF) at levels as low as 5 parts per billion in fish and 200 parts per trillion in water. Fish tissue extracts are subjected to gel permeation chromatography (GPC) and silica gel chromatography; water samples are extracted with dichloromethane. Analyses are carried out by gas chromatography in which either electron capture or thermionic specific detectors are used. The applicability of the methods is demonstrated by analyses of water and fish samples from exposure studies.

S,S,S-Tributylphosphorotrithioate (DEF) is widely used as a cotton defoliant in California and the southern United States. The U.S. Department of Agriculture reported that 3.4 million pounds of DEF were applied to 2.3 million acres of cotton in 1976 (1). The widespread use of this compound suggests the possibility for contamination of freshwater fisheries from runoff and possibly from aerial transport (2). Studies conducted at the Columbia National Fisheries Research Laboratory were designed to determine the toxicity of DEF to fish and fish food organisms.

The defoliant DEF has been shown to be acutely toxic to fish and aquatic invertebrates. Acute toxicity studies indicate 96-h LC_{50} (concentration in water producing 50% mortality) values of 660 μg/L for rainbow trout (*Salmo gairdneri*), 620 μg/L for bluegills (*Lepomis macrochirus*), 100 μg/L for an amphipod (*Gammarus fasciatus*), and 2100 μg/L for a stonefly (*Pteronarcys*) (3). Furthermore, compounds of this class, the organophosphorothioates, are known to produce delayed neurotoxic effects in a variety of animal species (4). Specifically, DEF has been shown to produce delayed neurotoxicity in hens (5). Recent, unpublished studies at the Columbia Laboratory have demonstrated that DEF is extremely toxic to rainbow trout and channel catfish (*Ictalurus punctatus*) under chronic exposure conditions, reducing growth and survival at concentrations below 10 ppb. The methods presented herein were developed to provide residue data in support of these chronic toxicity studies.

Although various satisfactory methods exist for extraction and cleanup of DEF from cottonseed (6, 7), produce (8), and fats (9), each involves a more extensive sample manipulation than was deemed practical for our analytical operations dealing with more than 100 samples of fish. A simple GPC enrichment step was demonstrated in this laboratory to separate lipids and biogenic material from DEF. Subsequent chromatography on silica gel provided the final step in an enrichment procedure which was most economical in terms of materials and time demands.

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Experimental

Reagents and Apparatus

(a) *Organic solvents.*—Glass distilled (Burdick & Jackson Laboratories, Inc., Muskegon, MI).

(b) *Silica gel.*—EM-60, 70–230 mesh (E. Merck, Darmstadt, GFR); activated 24 h at 130°C.

(c) *Sodium sulfate.*—Mallinckrodt, Inc., St. Louis, MO; heated 8 h at 500°C before use.

(d) *Bio-Beads SX-3 GPC resin.*—200–400 mesh (Bio-Rad Laboratories, Richmond, CA).

(e) *DEF standard.*—Mobay Chemical Corp., Kansas City, MO.

Gel Permeation Chromatography

GPC was performed using a GPC Autoprep Model 1001 (ABC Laboratories, Columbia, MO) equipped with a 2.5 cm id × 60 cm glass column (Kontes, Vineland, NJ), packed with 60 g Bio-Beads SX-3 to a bed depth of 32 cm in cyclohexane–dichloromethane (95 + 5) (solvent A).

Gas-Liquid Chromatography

GLC analyses were carried out using a Varian chromatograph, Model 3700 (Varian Associates Inc., Walnut Creek, CA), equipped with either a

linearized electron capture (EC) detector or a thermionic specific (TS) detector.

Gas chromatographic conditions used with the EC detector were as follows: 1.8 m × 2 mm id glass column packed with 3% OV-17 on 100–120 mesh Supelcoport; nitrogen carrier gas at 20 mL/min; temperature program of initial temperature 200°C for 2 min, temperature ramp 3°/min to 230°C, and hold 1 min. Operating conditions used with the TS detector were as follows: 1.5 m × 2 mm id glass column packed with 3% SE-30 on 80–100 mesh Chromosorb W(HP); nitrogen carrier gas at 14 mL/min; temperature program of initial temperature 180°C for 6 min, temperature ramp 3°/min to 210°C, and hold 6 min. Flow conditions for TS detector were 4.0 mL hydrogen/min and 175 mL air/min. Either column provided sufficient resolution for the determination of DEF and columns were used interchangeably.

Sample Preparation

Ten g samples (wet weight) of bluegills were homogenized and mixed with anhydrous Na_2SO_4 (4:1 Na_2SO_4:fish), and this mixture was allowed to equilibrate 2–3 h. The resulting dry mixture was blended using an Oster blender (Oster Corp., Milwaukee, WI) to the consistency of a dry powder. Each 50 g sample was subsequently spiked with a solution of DEF in methylene chloride (CH_2Cl_2) at the desired level. Water samples (50 mL) were fortified to the desired level by adding a solution of DEF in acetone.

Analytical Procedure

All glassware was rinsed with 1M HCl, dried, and rinsed twice with CH_2Cl_2 before use.

Water samples (50 mL) were placed in 125 mL separatory funnels, buffered to pH 4 with 1 mL 5% acetate buffer, and extracted with three 20 mL portions of CH_2Cl_2. Extracts were combined and dried with anhydrous Na_2SO_4. One mL of nonane was added and the CH_2Cl_2 was removed by rotary evaporation. The residue was quantitatively transferred to a screw-top test tube and diluted, if necessary, to produce a DEF concentration of approximately 0.025 μg/mL. Processed water samples were analyzed by EC-GLC.

Fifty g quantities of the mixture of fish and Na_2SO_4(1:4) were poured into an extraction column (2.5 cm id × 35 cm) containing a 5 cm bed of anhydrous Na_2SO_4 above a plug of glass wool. A 5 cm bed of anhydrous Na_2SO_4 was added to the top of the mixture. The sample container was rinsed with two 10 mL portions of CH_2Cl_2, and these washes were applied to the column. The sample was subsequently extracted with an additional 100 mL CH_2Cl_2, with the column flow rate adjusted to ca 1 mL/min. The eluate was collected in a 300 mL round-bottom flask and reduced in volume to ca 1 mL by rotary evaporation. The solution was quantitatively transferred to a screw-top test tube, using CH_2Cl_2. The CH_2Cl_2 was removed using a stream of dry nitrogen. The extract was then diluted to 7.0 mL with solvent A, and the resulting solution was agitated and then centrifuged to remove particulate matter.

DEF was separated from lipid material by GPC. A 5 mL loop was used to introduce the sample, in solvent A, onto the GPC column. The eluting solvent (solvent A) was delivered at a flow rate of 5 mL/min. The initial 130 mL eluate (containing lipid material) was discarded, and the following 40 mL fraction was collected in a round-bottom flask. One mL toluene was added to the flask, and the solvent volume was reduced by rotary evaporation to ca 1 mL.

Following GPC, the sample was then quantitatively transferred, using 2% acetone in hexane, to a glass column (30 cm × 12 mm id) containing a small plug of glass wool and 40 g silica gel. A total of 30 mL (including transfer rinses) 2% acetone in hexane was applied to the column, and the eluate was discarded. Subsequently, 30 mL 5% acetone in hexane was applied to the column, and the eluate was collected in a round-bottom flask. One mL toluene was added to the flask and the solvent volume was reduced to about 1 mL by rotary evaporation. The solution was quantitatively transferred to a 10 mL screw-top test tube, using acetone. The solvent volume was reduced under a stream of nitrogen to ca 1 mL and then adjusted to the final volume with toluene.

Results and Discussion

The recovery data presented in Tables 1 and 2 demonstrate that DEF can be reproducibly determined in samples of water and of fish tissue. The recovery data for both spiked water and spiked fish samples appear to fall into 2 distinct groups. The data sets produced during the initial validation of the 2 methods (designated by the footnotes in Tables 1 and 2) are characterized by generally lower recoveries than those generated in the quality control protocol included in the analyses of water and fish from the exposure studies. No data are available to indicate

Table 1. Recovery data for DEF from spiked fish samples

Spiking level, ppb	Mean rec., %	SD	n
10[a]	66	4.7	6
10	94	10.2	3
100[a]	72	3.0	6
100	83	12.7	3
200	97	4.7	5
1000[a]	71	4.9	6
1000	95	13.8	5
4000	93	8.2	3

[a] These data were generated in the validation study; the remaining data were generated from quality control samples included in the analysis of samples from the exposure study.

Table 2. Recovery data for DEF from spiked water samples

Spiking level, ppb	Mean rec., %	SD	n
1.6	101	8	3
3.8	92	8	3
9.0	105	4	3
10.0[a]	70	12	6
20.0	96	8	3
30.0	103	1	3
100.0[a]	92	6	6
500.0[a]	83	14	6

[a] These data were generated in the validation study; the remaining data were generated from quality control samples included in the analysis of samples from the exposure study.

the cause of the lower recoveries from the validation studies.

Chromatographic analyses by EC-GLC of processed extracts of fish tissue spiked at 100 ppb and 1000 ppb demonstrate that the determination of DEF at these levels was not impeded by background interferences. However, at the lowest spiking level (10 ppb), background interferences equivalent to 1–2 ppb must be subtracted from observed DEF values. Thus, the minimum detection limit was estimated to be 5 ppb in spiked fish (bluegill) tissue, based on a required signal-to-noise ratio of three.

These methods have been used in the determination of DEF in water and fish samples generated in chronic toxicity studies carried out at the Columbia laboratory. Water samples, containing relatively low levels of interferences, were successfully analyzed by GLC, using the electron capture detection technique. Representative chromatograms of EC-GLC analyses of water samples from the exposure experiments (Figure 1) demonstrate the applicability of the method for determining DEF in water at concentrations as low as 200 ppt. This estimate of the value for the method lower limit was made from comparison of the maximum background signals associated with unspiked water samples with those from sub-ppb spiked samples. A signal intensity of 3 times background was assigned as the minimum required for a positive determination.

Gas chromatographic analyses of processed fish from the exposure studies could usually be performed using either EC or TS detectors to provide reliable DEF residue values as low as 10 ppb and lower. In some samples of fish, background components interfered with determination of DEF below 10 ppb, particularly in analyses using the TS detector. Representative gas chromatographic analyses of fish samples containing bioconcentrated DEF are shown in Figure 2. The data presented in chromatograms D, E, and F of Figure 2 are representative of TS-GLC analyses of processed fish samples. The analysis of a fish sample shown in chromatogram F of Figure 2 is presented as a worst-case situation for analyses using the TS detector. Normally, the interfering component is observed by TS-GLC as a small shoulder on the trailing end of the DEF peak as shown in chromatogram E of Figure 2. The variable nature of this interference generally yields a method lower limit of greater than

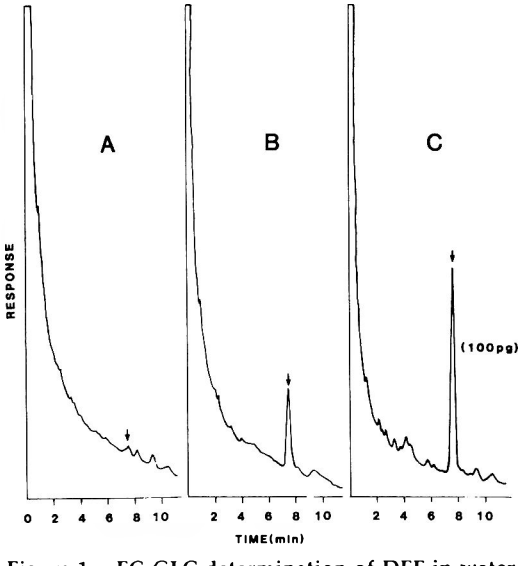

Figure 1. EC-GLC determination of DEF in water samples from a chronic exposure study: A, control; B, 0.6 ppb residue; C, 3.0 ppb residue.

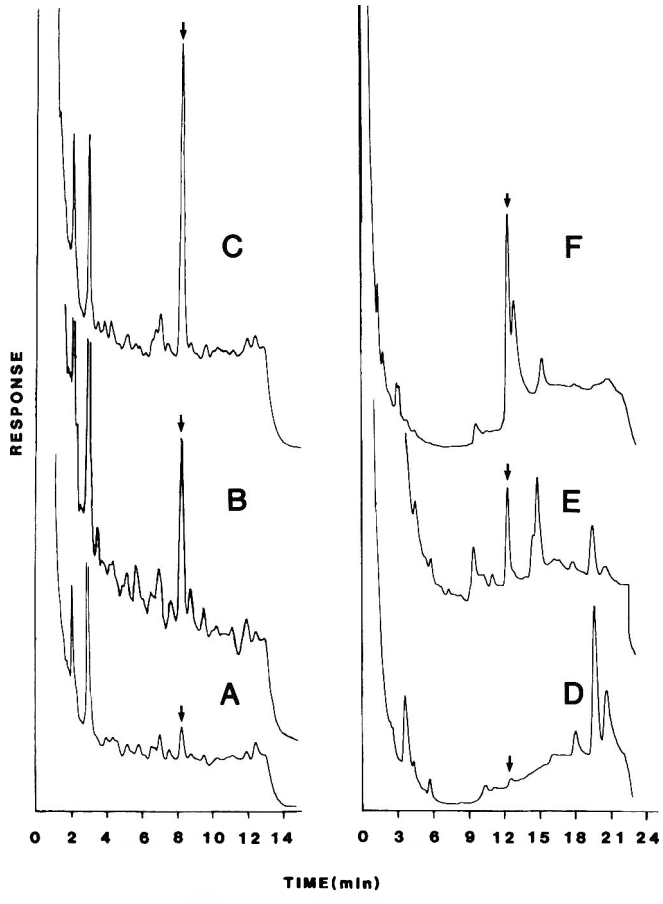

Figure 2. Determination of DEF in fish samples from a chronic exposure study: A and D, unexposed fish; B and E, 10 ppb residue; C and F, 20 ppb residue. A, B, and C, EC-GLC; D, E, and F, TS-GLC.

10 ppb when TS-GLC is used. The identity and source of the interfering component are unknown, but the higher levels were encountered only in the larger fish from later stages of the exposure study. These analytical methods in conjunction with toxicity data should be of value in assessing the potential impact of DEF on freshwater ecosystems.

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High Resolution Gas Chromatography of Chlorinated Benzenes

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Chlorinated benzenes have been found as contaminants in foods and water. These high production volume chemicals may enter the environment and food chain through improper waste disposal, use as solvents and odor control agents, or as impurities in other industrial chemicals. Because of differences in the electron capture responses of the isomers at each chlorination level, residue quantitation requires the separation of all 12 chlorobenzenes. Resolution studies were made on packed and capillary columns coated with Kovats' $C_{87}H_{176}$ hydrocarbon, OV-101, OV-210, OV-17, and Carbowax 20M. Satisfactory resolution of all 12 chlorobenzenes was obtained with a Carbowax 20M-coated 20 m × 0.25 mm id capillary column operated isothermally at 120°C.

Over the past few years, there have been increasing awareness and concern that foods can be contaminated with toxic industrial chemicals. As illustrated by polychlorinated biphenyls, industrial chemicals whose uses are not directly associated with food production or processing have been found in foods. Because approximately 50 000 industrial chemicals are manufactured annually in the United States, the Food and Drug Administration (FDA) uses criteria described by Jelinek (1) to identify those with a high potential for endangering the food supply. The factors considered in selecting industrial chemicals for investigation include production volume, toxicity, toxic by-products, solubility behavior, environmental stability, patterns of end use, and means of disposal.

Based on these factors, chlorinated benzenes are high priority candidates for investigation as food contaminants. Over 300 million pounds of monochlorobenzene and 140 million pounds of 1,2- and 1,4-dichlorobenzenes were produced in the United States in 1979 (2), mainly for use as solvents, chemical intermediates, space deodorants, and for moth control (3). Production estimates by the U.S. Environmental Protection Agency (EPA) for several higher chlorinated benzenes used primarily as chemical intermediates, solvents, dye carriers, and dielectric fluids are in the millions or tens of millions of pounds per year (4). Additionally, several million pounds of hexachlorobenzene (HCB), some of which may be recovered for use as a fungicide, are generated annually as by-product waste in the manufacture of perchloroethylene (5). Improper waste disposal practices and some of the major uses for chlorobenzenes provide direct routes for the entry of these chemicals into the ecosphere. Their lipophilic nature and their stability to hydrolysis, oxidation, and biodegradation can lead to bioaccumulation in the food chain and persistence in the environment. An EPA report on health effects testing needs for chlorobenzenes (4) indicates that 98 million to 162 million pounds of monochlorobenzene, chiefly that used as a herbicide formulation solvent, and over 50 million pounds of dichlorobenzenes are released annually into the environment. The EPA report states that HCB has been shown to be oncogenic in 2 animal species and that considerable evidence suggests that other chlorinated benzenes may also be oncogenic.

Monochloro- through hexachloro-substituted benzenes ranging in levels from trace to 6.7 ppm were found by the FDA Division of Chemical Technology in freshwater fish from several U.S. locations (6). In a 1975 survey for suspected carcinogens in drinking water, EPA sampled finished water from 10 U.S. cities that use various types of raw water sources and water treatment processes; of the 10 samples analyzed in the survey, 9 contained monochlorobenzene, 4 contained at least 2 dichlorobenzenes, and 1 contained a trichlorobenzene isomer (7). Veith et al. (8) identified Cl_2- through Cl_6-benzenes in fish from several major U.S. watersheds.

Because all 12 chlorinated benzenes might be found in foods or environmental samples, it would be useful to have the ability to separate all the congeners. This work reports our attempts and success in developing a gas chromatographic system that accomplishes this goal.

Experimental

Reagents and Apparatus

(a) *Chlorinated benzenes.*—The chlorinated benzenes used in this study were from the FDA Industrial Chemical Repository, Division of Chemical Technology, FDA, Washington, DC 20204. All 12 chlorobenzene congeners are

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commercially available from Aldrich Chemical Co., Inc., Milwaukee, WI 53233 and RFR Corp., Hope, RI 02831.

(b) *Gas chromatograph.*—Hewlett-Packard Model 5710 equipped with constant current ^{63}Ni electron capture detector (ECD) and 6 ft (1.8 m) × 4 mm id coiled glass columns. Columns packed with 80–100 mesh Chromosorb W(HP) containing 5% OV-101, 15% OV-210, or 10% OV-17 were conditioned overnight at 280–300°C with ca 60 mL/min carrier gas. Column packed with 10% Carbowax 20M on 100–120 mesh Chromosorb W(HP) was conditioned overnight at 200°C with ca 60 mL/min carrier gas. Operating conditions: temperatures (°C)—columns 120–140 (see Results and Discussion), injector 250, detector 300; argon–methane (95 + 5) carrier gas 60 mL/min; recorder—span 1 mv full scale (10 in.), chart speed 0.25 in./min.

(c) *Gas chromatograph.*—Varian Model 3700 equipped with constant current ^{63}Ni ECD and flame ionization detector (FID), and 0.25 mm id wall-coated open tubular (WCOT) glass capillary columns (see Table 1 for liquid phases and column lengths). Operating conditions: temperatures (°C)—columns 120, injector 250, detectors 320; FID gas flows—hydrogen 30 mL/min, air 300 mL/min; ECD and FID nitrogen make-up 20 mL/min; recorder—span 1 mv full scale (10 in.), chart speed 0.25 in./min; FID electrometer sensitivity (range 1, attenuation 1) 1×10^{-12} amp full scale. Hydrogen carrier velocities and split flows are given in Table 1.

Procedure

The chlorinated benzenes were weighed, dissolved, mixed, and serially diluted with isooctane to yield the desired concentrations. Three microliter aliquots were injected into the gas chromatograph.

Results and Discussion

Separation of a mixture of the 12 chlorinated benzenes on several packed gas-liquid chromatographic columns was investigated. Attempted separations on 5% OV-101 and 15% OV-210 columns operated at 120°C are illustrated in Figure 1. Two pairs of isomers, the 1,3- and 1,4-dichlorobenzenes and the 1,2,3,5- and 1,2,4,5-tetrachlorobenzenes, are not resolved on either column. Separation of the same mixture was attempted on 10% OV-17 and 10% Carbowax 20M columns operated isothermally at 140°C. As shown in Figure 2, the OV-17 column failed to separate either the 1,3- and 1,4-dichlorobenzenes or the 1,2,3,5- and 1,2,4,5-tetrachlorobenzenes. Although the Carbowax column did separate the dichlorobenzenes, it only partially resolved the tetrachlorobenzenes. Attempts to improve separation of the tetrachloro pair by lowering the temperature resulted in unacceptably long analysis time, without complete resolution of the compounds.

Table 1. Gas-liquid chromatography of chlorinated benzenes at 120°C in 0.25 mm id WCOT capillary columns containing selected liquid phases

Liquid phase	Column length, m	Hydrogen carrier velocity, cm/s	Split flow, mL/min
Carbowax 20M	20	61.5	71
$C_{87}H_{176}$	20	41.7	40
OV-17	35	44.0	40
OV-101	40	29.2	38
OV-210	43	39.0	120

In order to increase resolution, capillary column chromatography was investigated. Chromatograms obtained at 120°C with WCOT capillary columns containing 5 different liquid phases are shown in Figure 3. The OV-210 capillary did not completely separate the 1,3- and 1,4-dichlorobenzenes or the 1,2,3,5- and 1,2,4,5-tetrachlorobenzenes. Kovats' $C_{87}H_{176}$ liquid phase (9, 10) was tried because it had successfully separated all 19 chlorinated anisoles (11). However, it did not even partially resolve the 1,3- and 1,4-dichlorobenzenes or the 1,2,3,5- and 1,2,4,5-tetrachlorobenzenes. The OV-101 capillary column almost completely resolved the dichloro isomers, but did not satisfactorily separate the tetrachloro isomers. Complete resolution of all 12 chlorobenzenes was achieved with both the OV-17 and the Carbowax 20M WCOT columns. The separations were accomplished in under 1 h on OV- 17 and in less than $^1/_2$ that time on Carbowax 20M. Because the original chromatograms were reduced for publication, the individual peaks for dichlorobenzenes in Figure 3 are not readily discernible and are not labeled. As shown in enlarged scale in Figure 4, the dichlorobenzenes elute from both the OV-17 and Carbowax 20M WCOT columns in the order: 1,3-, 1,4-, and 1,2-dichlorobenzene.

ECD and FID responses for the chlorinated benzenes (Table 2) were calculated in terms of the peak height (cm) per ng of compound chromatographed in a 15 m segment of the Carbowax 20M WCOT column that gave the separations shown in Figures 3 and 4. (Although the col-

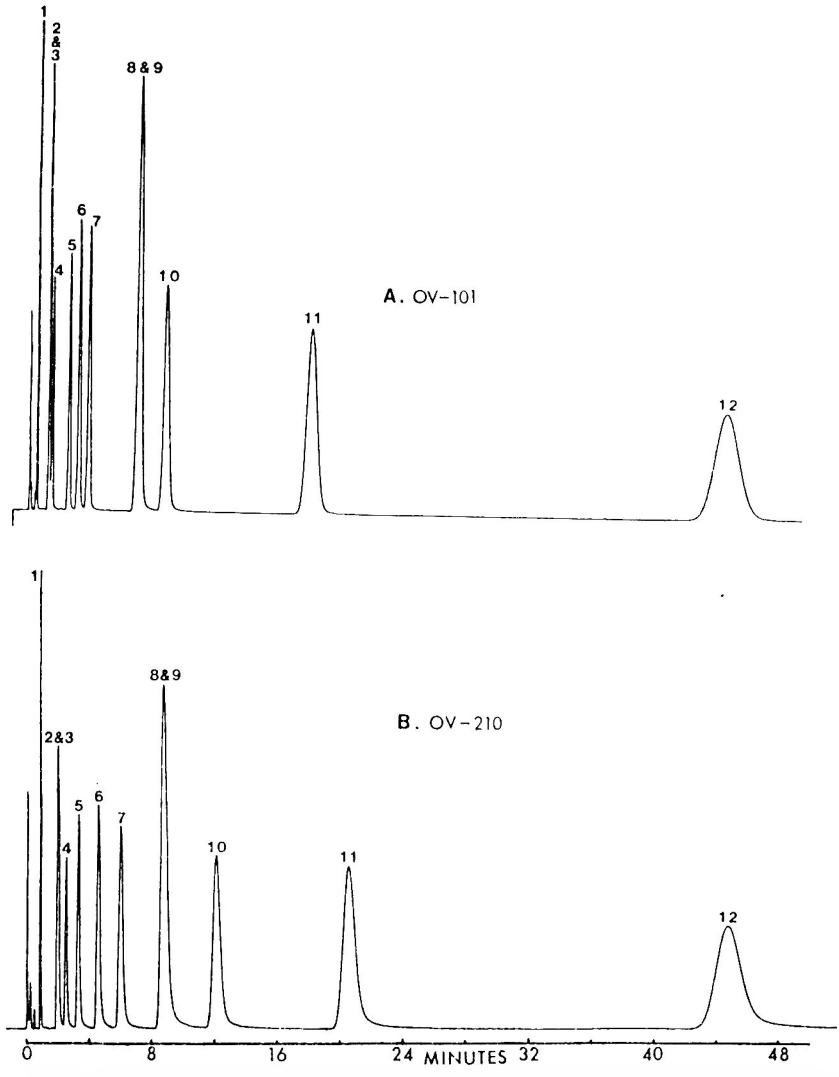

Figure 1. Electron capture gas-liquid chromatograms of chlorinated benzenes at 120°C in 6 ft × 4 mm id glass columns containing 80-100 mesh Chromosorb W(HP) coated with A, 5% OV-101; B, 15% OV-210. See Table 2 for peak identification.

umn was shortened by 25%, it continued to resolve all 12 chlorobenzene congeners and it did so in about 20 min at 120°C with hydrogen carrier at 52 cm/s.) Peak height was used as the measure of the detector response because an integrator capable of measuring the areas of the narrow peaks in the capillary column chromatograms was not available. Ratios of ECD response factors (peak height × ECD attenuation/ng) to FID response factors (peak height × FID attenuation/ng) for the chlorinated benzenes were calculated to determine the relative responses of the 2 detectors (at equivalent attenuation) for each congener and to provide a rough approximation of the electron capture relative response values that would have been obtained for the 12 chlorobenzenes from peak area measurements. The ECD:FID response ratios are given in the last column of Table 2.

The approximation of a peak area function from peak height data (in effect, cancelling out the bias for peak height response factors to decrease with increasing peak width and retention time) requires an explanation. It was possible in this study because (a) the ECD and FID analyses of the chlorobenzenes were carried out with the same chromatographic column at the same temperature and hydrogen carrier velocity so

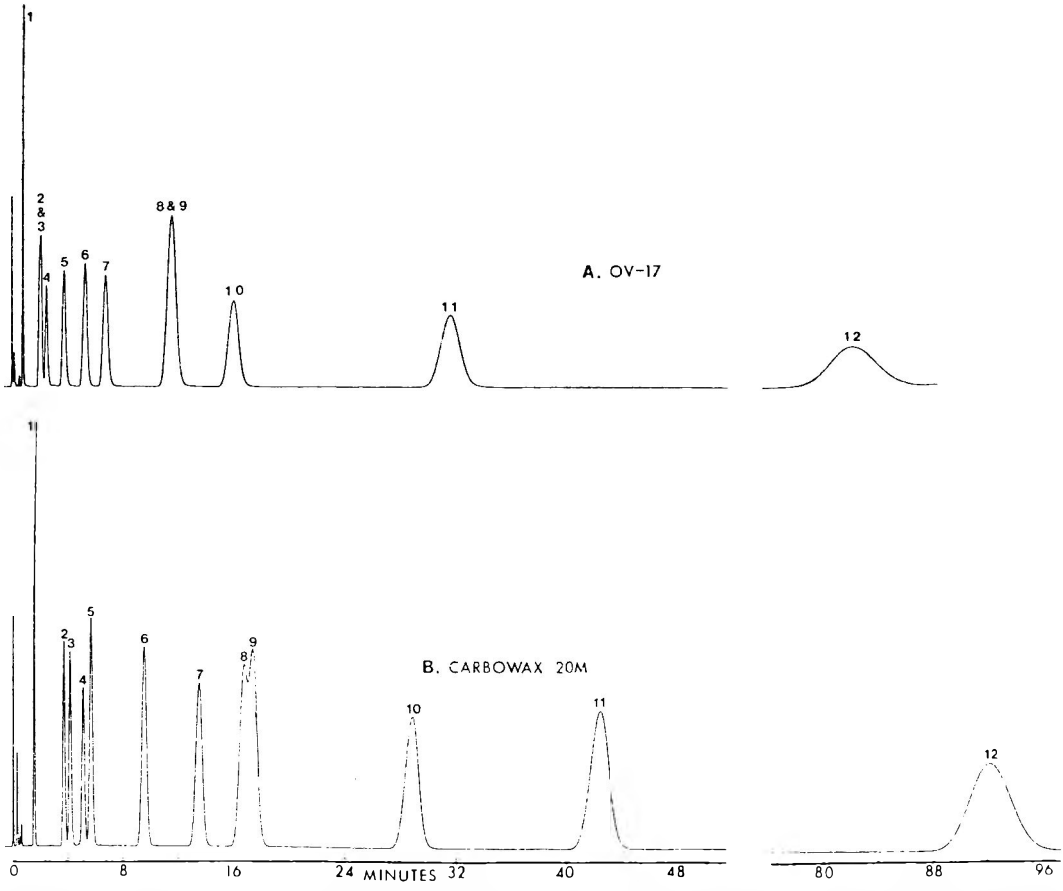

Figure 2. Electron capture gas-liquid chromatograms of chlorinated benzenes at 140°C in 6 ft × 4 mm id glass columns packed with A, 10% OV-17 on 80-100 mesh Chromosorb W(HP); B, 10% Carbowax 20M on 100-120 mesh Chromosorb W(HP). See Table 2 for peak identification.

that the ECD and FID responses for an individual congener were virtually identical in peak shape; (b) it was evident from the chromatograms that the peak areas obtained per ng of the 12 chlorobenzenes varied much more with the ECD than the FID; and (c) the steady decline in FID peak height/ng as the retention time increased appeared to reflect the aforementioned bias in response factors based on peak height. In support of items b and c, it should be noted that absolute responses of the FID for benzene, chlorobenzene, 1,3-dichlorobenzene, and 1,2,4-trichlorobenzene have been reported on both a weight and a molar basis by Koragozler and Simpson (12). Although they found that the FID response in terms of coulombs/g or coulombs/mole decreased as the number of chlorine atoms in the molecule increased, the total reduction after addition of 3 chlorine atoms to the molecule was only about 20% more than could be attributed solely to the corresponding reduction in the carbon content of the molecule. Thus, as the number of chlorine atoms in the molecule increases, the absolute response of the FID decreases by a very much smaller amount than the increase in the peak area response of the ECD. Therefore, the ratios of ECD response to FID response should provide a reasonable representation of ECD (peak area) relative responses for the chlorobenzenes.

The ECD:FID response ratios in Table 2 show that monochlorobenzene gives much less electron capture response than the other congeners. As the chlorine substitution level in the compound increases, the electron capture response increases markedly at first (about 100× at the Cl_2 level, 10× at the Cl_3 level) and then continues to rise more slowly. The positions of the chlorine atoms in the molecule have such a strong effect that 1,2,3-trichlorobenzene gives greater electron capture response than 1,2,4,5-tetrachlorobenzene

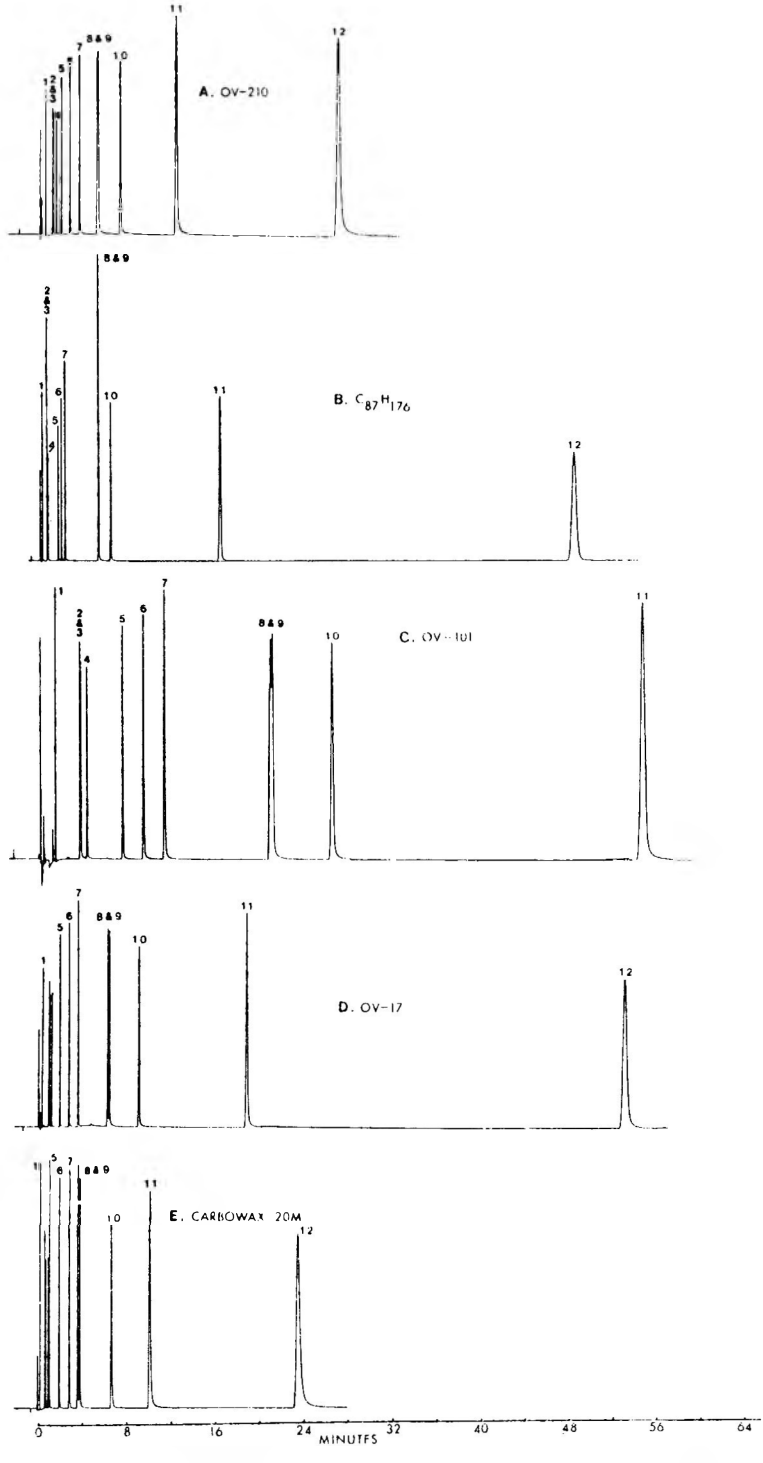

Figure 3. Electron capture gas-liquid chromatograms of chlorinated benzenes at 120°C in WCOT capillary columns containing A, OV-210; B, Kovats' $C_{87}H_{176}$; C, OV-101; D, OV-17; E, Carbowax 20M. See Table 2 for peak identification.

Table 2. High resolution gas-liquid chromatography of chlorinated benzenes: ECD and FID response data [a]

Peak	Chlorinated benzene	Detector response, peak ht (cm/ng[b]) FID[c]	ECD[d]	ECD:FID[e] response ratio
1	monochloro	9.68	0.15	0.31
2	1,3-dichloro	5.50	11.9	43.4
3	1,4-dichloro	5.31	4.7	17.6
4	1,2-dichloro	4.82	8.1	33.6
5	1,3,5-trichloro	3.73	61.7	330
6	1,2,4-trichloro	2.54	35.1	276
7	1,2,3-trichloro	1.94	49.9	514
8	1,2,3,5-tetrachloro	1.65	64.9	786
9	1,2,4,5-tetrachloro	1.55	31.3	404
10	1,2,3,4-tetrachloro	0.96	44.9	936
11	pentachloro	0.57	59.4	2084
12	hexachloro	0.22	36.5	3318

[a] Data from gas-liquid chromatography of a mixture of 12 chlorobenzenes in a 15 m × 0.25 mm id Carbowax 20M WCOT capillary column at 120°C with hydrogen carrier velocity of 52 cm/s and split flow of 109 mL/min. ECD and FID responses were determined in separate runs with the column connected to 1 detector at a time.

[b] Maximum deflection (cm) of recorder pen per ng compound injected into the WCOT column via a splitter operating at a split ratio of 72:1.

[c] FID electrometer sensitivity at 32×10^{-12} amp full scale, i.e., range 1, attenuation 32.

[d] ECD controller attenuation at 10 × 64.

[e] Ratio of ECD response factor (cm peak ht × attenuation/ng) to FID response factor (cm peak ht × attenuation/ng) for the compound.

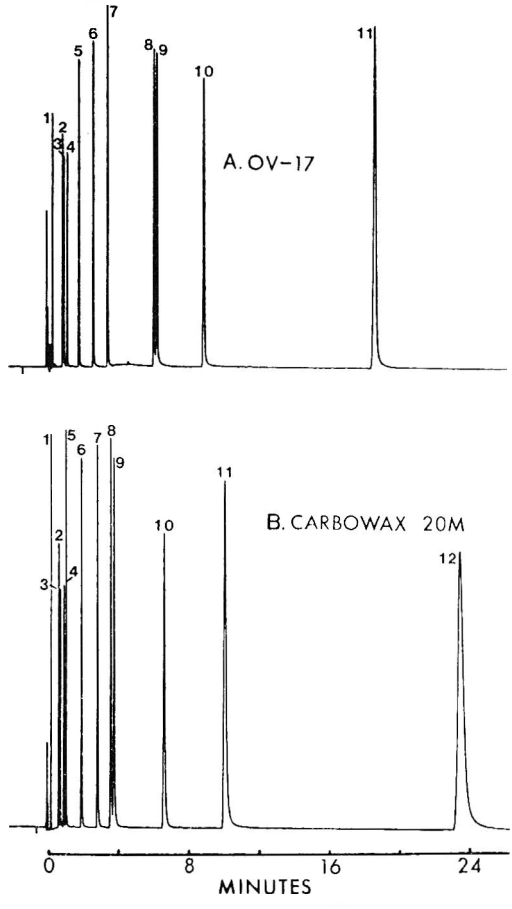

Figure 4. Electron capture gas-liquid chromatograms of chlorinated benzenes at 120°C in A, OV-17 WCOT capillary column (see Figure 3D for complete chromatogram); B, Carbowax 20M WCOT capillary column. See Table 2 for peak identification.

despite the higher level of chlorine in the latter. Within each group of 3 compounds at the same chlorination level, one isomer produces about ½ the ECD response of the other isomers. Because 1 compound in each of the 2 difficult-to-separate pairs (i.e., the 1,3- and 1,4-dichlorobenzenes and the 1,2,3,5- and 1,2,4,5-tetrachlorobenzenes) gives only ½ the ECD response of the other compound in that pair, the differences between the responses could result in incorrect quantitation of an inadequately resolved residue. If, for example, a residue of 1,3-dichlorobenzene was misidentified as a result of inadequate chromatographic resolution and its concentration was determined by electron capture gas-liquid chromatography with 1,4-dichlorobenzene as the analytical standard, the error in identification would be compounded by an error of more than 100% in the residue level.

Because environmental samples may contain all 12 chlorinated benzenes and because the ECD sensitivity differs for the various isomers, it is imperative that all of the compounds be resolved if accurate quantitation is to be assured. This work has shown that a 15 or 20 m × 0.25 mm Carbowax 20M WCOT capillary column operated isothermally at 120°C is sufficient to accomplish the required separation. Additionally, a 35 m × 0.25 mm OV-17 WCOT capillary at 120°C also resolves all the chlorobenzenes, albeit in twice the time required with the Carbowax 20M column. These columns can be used for confirmatory analysis because their polarities are markedly different.

The work published here was presented at the 93rd Annual Meeting of the Association of Official Analytical Chemists (13). Since then, Oliver and Bothen (14) have also reported that all 12 chlorobenzenes are separated on a Carbowax 20M WCOT capillary column.

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Extraction and Cleanup Procedures for Determination of Diarylphosphates in Fish, Sediment, and Water Samples

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Methods for determination of triaryl/alkylphosphates (TAPs) in water, fish, and sediment have been extended to determination of the diarylphosphate (DAP) degradation products. DAPs were extracted from water (adjusted to pH 0.5) by use of XAD-2 resin and determined by gas-liquid chromatography as butyl esters. Recovery of diphenylphosphate (DPP) and *o*-, *m*-, *p*-dicresylphosphates (DoCP, DmCP, DpCP) were >95% in water samples fortified at 1, 10, and 50 μg/L. DAPs were extracted from fish with methanol and the extracts were cleaned up on reverse phase (C18) silica cartridges. Recoveries were >87% for DPP, DoCP, DmCP, and DpCP in fish muscle fortified at 50, 100, and 500 ng/g. Sediments were refluxed with aqueous methanol and DAPs were recovered by use of XAD-2 resin. Recoveries of DAPs from sediments fortified at 50 and 100 ng/g were >76%. Interferences (1-10 ng/g) from phosphorus or nitrogen-containing GLC peaks prevented sub-ng/g level analysis for DAPs in sediment and fish extracts.

Diphenylphosphate (DPP) has been identified as a major degradation product of triphenylphosphate in water (1) and sediment (2) and of 2-ethylhexyldiphenylphosphate (EHDP) in fish (3). Other triaryl- and triaryl/alkylphosphates (TAPs) hydrolyze rapidly in alkaline solution to yield the corresponding diarylphosphates (DAPs) (4), which are more stable to hydrolysis than tri- or monoarylphosphate esters (5). Determination of DAPs is of interest for purposes of monitoring the fate of TAPs in the environment. Development of suitable methods for DAPs would also assist studies on the metabolism of TAPs by fish and mammals. Like the dialkylphosphates, DAPs are water-soluble and exist as anions at neutral pH (5). They are not extracted efficiently from water by partitioning with organic solvents and must be derivatized before gas chromatographic analysis. Howard and Deo (1) analyzed DPP semiquantitatively in natural waters (containing 15% NaCl and adjusted to pH 1-2) by extraction with ethyl ether and methylation of the extract for analysis by GLC. The purpose of this study was to extend procedures developed for the determination of TAPs (6, 7) in water, sediment, and fish samples to permit quantitative analysis for DAPs in environmental samples.

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Experimental

Reagents and Apparatus

Reagents, chromatographic materials, and apparatus were described previously (6). In addition, the following materials were used:

(a) *Diarylphosphates.*—^{14}C-(phenyl ring labeled) DPP was prepared as a byproduct of synthesis of 2-ethylhexyldiphenylphosphate (EHDP) (3). The product was purified by TLC using reverse phase TLC plates (Whatman KC18) and a solvent system of acetone–water (7 + 3). Specific activity of the purified product was 10.0 μCi/mg. Nonradiolabeled DPP was obtained from Eastman Organic Chemicals (Rochester, NY). Di(*o*-cresyl)-, di(*m*-cresyl)-, and di(*p*-cresyl)phosphate (DoCP, DmCP, DpCP) were prepared by hydrolysis of their corresponding tricresylphosphate analogs in 0.1N NaOH (24 h, 20°C) (8). The reaction mixtures were extracted with ethyl acetate to recover residual TAPs, then adjusted to ca pH 1 and re-extracted with ethyl acetate to recover to DAPs. The dicresylphosphate products were greater than 95% pure, based on their GLC response following butylation with diazobutane (relative to butyldiphenylphosphate). Stock solutions of the butylated derivatives of the dicresylphosphates and DPP were prepared in hexane (1.0 μg/mL). Solutions of underivatized DPP, DoCP, DmCP, and DpCP were prepared in methanol (100 μg/mL).

(b) *Water.*—HPLC grade (Baker Chemicals).

(c) *Macroreticular resin.*—XAD-2 resin (Rohm and Haas Ltd, Philadelphia, PA); prepared as described by Daughton et al. (9).

(d) *Chromatographic cartridges.*—C-18 bonded silica Sep-Pak (Waters Associates).

(e) *Diazobutane.*—Prepared daily from *N*-butyl-*N'*-nitro-*N*-nitrosoguanidine (BNNG), using procedure of Stanley (10). Stored at −10°C in Teflon-lined screw-cap test tube.

Caution: BNNG is a possible mutagen and cancer suspect agent.

(f) *GLC operating conditions.*—Butylated DAP compounds and TAPs were separated on 1.0 m × 2 mm id columns of 3% SP-2100 or 3% SP-2250 on 80–100 mesh Supelcoport.

Helium carrier gas 30 mL/min. Column oven 220°C (isothermal) or temperature-programmed from 100 to 220°C at 6°/min for both columns.

(g) *Measurement of ^{14}C-radioactivity.*—Fish and sediment samples were combusted, and $^{14}CO_2$ was collected on a Packard 306 oxidizer. Aliquots of fish, sediment, and water samples containing ^{14}C-DPP residues were assayed by liquid scintillation counting (LSC) and results were expressed as ng/g sample.

Recovery Studies

(a) Ground fish muscle (whitefish, *Coregonus clupeaformis*) (2.5 g) was fortified at 50, 100, and 500 ng/g with DPP (^{14}C-DPP), DoCP, DmCP, DpCP, EHDP, and TPP by pipetting a stock solution (methanol) containing all 6 compounds directly onto the fish tissue. Three to 6 replicates of each concentration were prepared.

(b) Rainbow trout (*Salmo gairdneri*, 150 to 300 g) were placed in aquaria containing dechlorinated tap water fortified with EHDP (^{14}C-labeled) at 60 μg/g. Following 10 h exposure, fish were removed and individual tissues were prepared for determination of DPP and EHDP content.

(c) River sediment (Red River near Winnipeg, Canada) was collected with an Ekman dredge and stored (−50°C) until use. Sediment (25 g wet weight) was fortified as described for fish at 50 and 500 ng/g (dry weight basis) in triplicate and allowed to stand 1 h before extraction. Sediment contained 2.3% organic carbon, 45% silt, 48% clay, 7% sand, and 50% water by weight.

To simulate degradation of TAPs under aerobic and anaerobic conditions, sediment (50 g wet weight) was fortified with ^{14}C-TPP and EHDP, 250 mL dechlorinated water was added, and the mixture was incubated under air or nitrogen aeration for 32 or 64 days. Sediment was separated from the overlying water by filtration (Whatman No. 1 paper) before extraction.

(d) Water (0.5 to 1 L) was fortified at 1, 10, and 50 μg/L with a stock solution (methanol) of DPP (^{14}C-DPP), DoCP, DmCP, DpCP, EHDP, and TPP. The water was stirred and allowed to stand 1 h before extraction.

METHOD

Extraction

Fish: Extract 2.5–5 g samples with methanol, using Polytron homogenizer described previously (6). Evaporate methanol extract to ca 2 mL, transfer residue to test tube, and reduce sample to ca 1 mL aqueous residue. Apply sample to C-18-chromatographic cartridge (pre-washed with acetone–water (7 + 3)). Elute cartridge with 4 mL acetone–water (7 + 3). Evaporate eluate to ca 1 mL, dilute with 5 mL 0.5N H_2SO_4, and saturate solution with NaCl. Extract aqueous phase with two 5 mL portions of ethyl acetate to recover DAPs and TAPs in organic phase. Dry ethyl acetate extract over anhydrous Na_2SO_4 and concentrate to ca 1.0 mL.

Sediment: Reflux samples (25–50 g wet weight) with 150 mL methanol–water (9 + 1) in flat-bottom flask for 16 h. Dilute aqueous methanol extract to ca 1 L with 0.5N H_2SO_4. Prepare column of XAD-2 resin (10 cm × 1 cm id). Wash column with 50 mL 0.5N H_2SO_4. Let dilute methanol extract pass through column (5–10 mL/min). When elution is complete, remove excess water by aspiration. Elute resin with 50 mL acetone. Evaporate acetone eluate to ca 2 mL, and transfer to test tube with 0.5N H_2SO_4. Extract acidified aqueous phase with ethyl acetate as described for fish.

Water: Adjust sample (1–10 L) to <pH 1 with H_2SO_4. For river water samples, filter through glass fiber filter (Whatman GFA) before pH adjustment and analyze suspended solids as described for sediment. Prepare column of XAD resin as described for sediment extracts and let water sample pass through column (5–10 mL/min). Recover DAPs and TAPs from the eluate as described for fish and sediment extracts.

Derivatization

Prepare diazobutane, using procedure of Stanley (10). Evaporate ethyl acetate solutions in screw-cap test tubes to ca 0.1 mL and add 1 mL diazobutane solution or until yellow color persists. Let reaction stand at room temperature (1 h); then remove ether and excess reagent by gentle evaporation under nitrogen. Avoid evaporation to dryness to prevent losses of butylated derivatives. Prepare small chromatographic column of acid alumina (6% deactivated with water) (6 cm × 6 mm id topped with anhydrous Na_2SO_4). Dilute derivatization mixture with hexane and apply sample to top of column. Elute with 15 mL hexane–diethyl ether

Table 1. Recovery of ^{14}C-DPP from water (60 μg/L, using XAD-2 resin)

pH	Volume, (L)	Radioactivity in each fraction,[a] % Acetone	Unextracted
0.5	0.1	88.1 ± 9.0	3.3 ± 1.0
	4.0	87.7 ± 9.5	—[b]
7.0	0.1	37.9 ± 20.6	3.7 ± 4.0

[a] Average of triplicate analyses ± standard deviation.
[b] Not determined. Radioactivity per mL was below detection limit.

(85 + 15). Discard first 9 mL eluate, which contains excess derivatization reagent. Collect remaining eluate in graduated centrifuge tube. Inject aliquot of extract into gas chromatograph equipped with SP-2250 column and compare peak area with area of corresponding butylated DAP or TAP standards. Confirm by analysis on SP-2100 column.

Results and Discussion

Radiolabeled DPP was recovered quantitatively from water (acidified to pH 0.5) by use of XAD-2 resin (Table 1). Adjustment of sample pH to less than 1 was essential for quantitative recovery of radioactivity. Unextractable radioactivity was determined by assaying aliquots of extracted water by LSC. XAD resin efficiently extracted DPP from water at pH 7.0 but the radioactivity could not be recovered from the resin with acetone unless acid conditions were used. Daughton et al. (9) observed similar pH-dependent recovery of some dialkylphosphates from water, using XAD-4 resin. Increasing the size of the water sample to 4 L resulted in recoveries similar to those at 0.1 L.

Derivatization of ^{14}C-DPP with diazomethane or diazobutane yielded single products. TLC of the products showed greater than 98% derivatization efficiency; no underivatized DPP could be detected by use of autoradiography. Butylated derivatives had the advantage of higher boiling points than methylated derivatives. This reduced losses on evaporation and allowed DAPs to be determined simultaneously with most TAPs, using isothermal column oven operation.

Recoveries of all 4 DAPs and TPP from water fortified at 1, 10, and 50 μg/L (Table 2) were greater than 95% at all concentrations. TPP was carried through the procedure as a check on the applicability of the method to TAPs. EHDP was not recovered as efficiently as TPP. Losses of EHDP were not investigated systematically but may have been due to hydrolysis of EHDP under the acid conditions used, although no increase in DPP concentration was observed. EHDP losses could also be due to volatilization during evaporation steps (before derivatization of DAPs). Nevertheless, the results indicate that di- and triarylphosphates can be determined in the same water sample by use of XAD resin column extraction.

Blank analyses of the water (HPLC grade) used in fortification studies indicated low level interfering peaks with retention times similar to butylated DPP (ca 0.3 μg/L), DmCP (ca 0.1 μg/L), and TPP (ca 0.1 μg/L) on both GLC phases (Figure 1). Possible sources of these interferences have been discussed in some detail by LeBel et al. (7) and Williams et al. (11). Preliminary work indicated that levels of interferences were substantially greater when concentrated HCl was used to lower the pH; thus H_2SO_4 was used to acidify all sample extracts. The derivatization

Table 2. Recovery of diarylphosphates (butyl esters) and triarylphosphates from water

Compound	Recovery at each concentration (μg/L),[a] % 1	10	50
DPP	102.0 ± 4.4	96.6 ± 16.0	112.0 ± 5.6
DoCP	103.8 ± 22.4	105.5 ± 15.3	105.7 ± 2.3
DmCP	96.7 ± 4.6	102.3 ± 19.7	102.0 ± 4.0
DpCP	99.3 ± 6.1	101.5 ± 18.7	95.7 ± 5.9
TPP	91.7 ± 3.9	98.4 ± 0.0	—[b]
EHDP	53.8 ± 8.3	40.0 ± 0.0	—

[a] 3 replicates at 1 and 50 μg/L, 4 replicates at 10 μg/L ± standard deviation.
[b] Not analyzed.

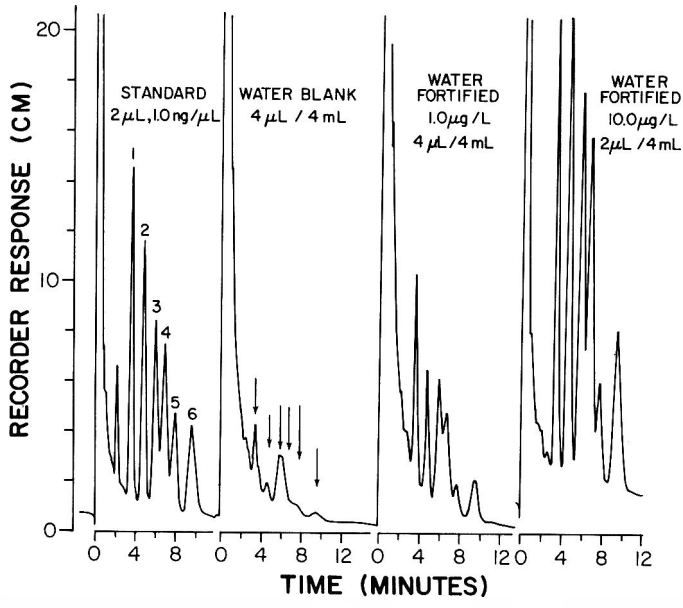

Figure 1. Chromatograms of fortified water sample extracts: analytical standard of butyl esters of 1, DPP; 2, DoCP; 3, DmCP; 4, DpCP as well as 5, EHDP; and 6, TPP, at 1.0 ng/μL concentrations, HPLC water (1 L) extract blank, water fortified at 1.0 μg/L, water fortified at 10.0 μg/L.

reagent contributed major early eluting peaks (Figure 2) in the chromatograms despite chromatographic cleanup on alumina. Detection limits in water for DAPs were limited by the reagent blanks to approximately 0.3 μg/L in 1 L samples. However, samples of 4 L tap water fortified at 0.6 μg/L with ^{14}C-DPP (Table 1) were passed through the column with little increase

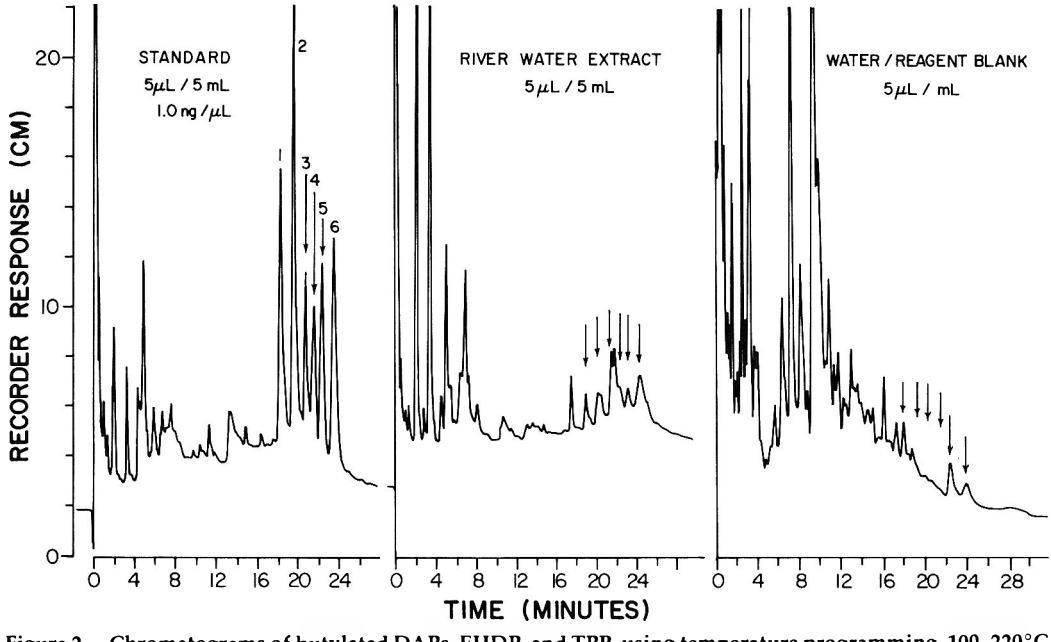

Figure 2. Chromatograms of butylated DAPs, EHDP, and TPP, using temperature programming, 100-220°C, 6°/min: analytical standard as in Figure 1; extract of river water sample from Winnipeg; HPLC water blank as in Figure 1 concentrated 5-fold.

Table 3. Recovery of DAPs (as butyl esters) and TAPs from fortified fish muscle

Compound	Recovery at each concentration (ng/g),[a] %		
	50	100	500
DPP[b]	87.3 ± 10.3	—[c]	88.7 ± 8.1
DoCP	110.7 ± 11.1	90.0 ± 17.3	100.0 ± 10.6
DmCP	97.2 ± 23.6	128.3 ± 30.1	96.7 ± 8.3
DpCP	125.3 ± 6.2	102.7 ± 33.6	100.0 ± 10.4
TPP	97.8 ± 8.8	103.3 ± 5.8	98.7 ± 14.7
EHDP	67.7 ± 9.8	—	79.3 ± 10.3

[a] Average of 3 or more replicates ± standard deviation.
[b] Recovery of ^{14}C-label determined by scintillation counting.
[c] Not determined.

in GLC background interferences, so detection limits could be lowered proportionally by use of larger sample sizes. Interferences could also be reduced by use of ethereal diazomethane prepared by distillation rather than the direct addition method used for preparation of diazobutane. Temperature programming of the GLC oven resulted in somewhat better resolution of the butylated dicresylphosphates (Figure 2), compared with isothermal operation (Figure 1).

The water extraction procedure was applied to the analysis of samples (4 L) from the Red River (Manitoba) taken within the City of Winnipeg and in a rural area upstream of the city. Aliquots of the sample extracts were chromatographed using temperature programming from 100 to 220°C (6°/min). (Figure 2). Results indicated low levels of contaminants in the samples with similar retention times to butylated diarylphosphates. Several of the peaks were not present in blank analyses using 1 L of HPLC-grade water (Figure 2). The actual identities of the peaks were not determined, although they are likely phosphorus-containing. TAPs had been previously found at lower levels (4.3–8.6 ng/L of tricresylphosphates and TPP, respectively) in tap water which was obtained from the Red River (12). Chromatograms of samples from urban areas contained similar profiles to those from the rural sampling area.

Recoveries of 4 DAPs, EHDP, and TPP from fortified fish muscle tissue are shown in Table 3. Recoveries were generally greater than 90%, ex-

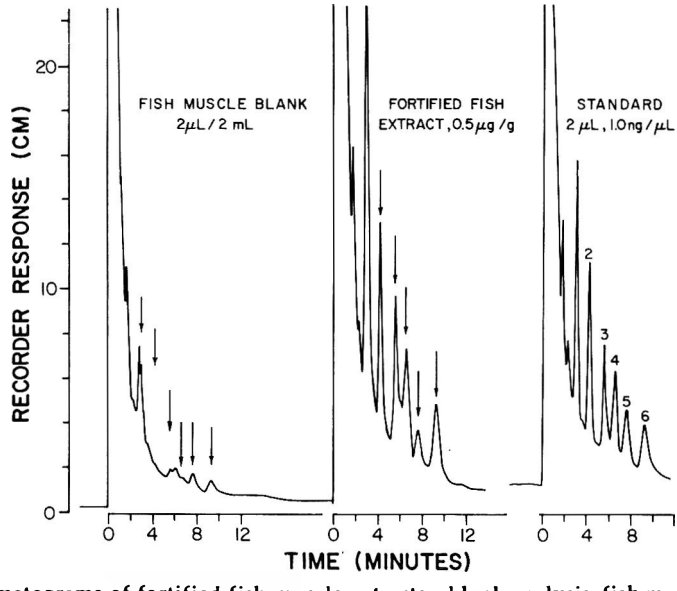

Figure 3. Chromatograms of fortified fish muscle extracts: blank analysis; fish muscle fortified at 0.5 μg/g; analytical standard as in Figure 1.

Table 4. Recovery[a] of ^{14}C-EHDP and DPP from fish tissues following exposure of fish to EHDP

Tissue	Time, h	EHDP,[b] ng/g	DPP,[b] ng/g	Not identified,[c] ng/g	Unextractable,[c] ng/g
Muscle	4 uptake	0.36	<0.01	<0.01	0.01
	10 uptake	0.31	<0.01	<0.01	0.01
	12 depuration	0.11	0.08	0.19	0.01
	36 depuration	0.07	0.04	<0.01	<0.01
Liver	4 uptake	3.11	1.17	5.92	0.41
	10 uptake	1.04	1.18	0.96	0.11
	12 depuration	0.41	0.72	0.57	0.11
	36 depuration	0.26	0.10	0.94	0.06

[a] Average of duplicate sample analyses.
[b] Determined by GLC.
[c] Determined by combustion and expressed as equivalents of EHDP by converting DPM/g to ng/g, using specific activity of EHDP.

cept for ^{14}C-DPP which averaged 88% and EHDP which averaged 73.5%. Recoveries of DPP determined by GLC of the butyl ester were higher because of a co-eluting interference which contributed to peak area (Figure 3). The co-eluting peak was also present in the reagent blank (homogenization without fish sample). The fish (whitefish) was obtained from a freshwater lake in northern Manitoba, a region likely free of significant TAP contamination in natural waters. Use of XAD resin as an initial extraction step for the fish extract (after evaporation of methanol) was also investigated but was unnecessary for the fish samples because it did not significantly improve recoveries or reagent blanks. Omitting the derivatization step in analysis for DAPs permits rapid analysis for TAPs in fish tissues, using this procedure.

Recovery of ^{14}C-DPP from fish tissue was investigated to determine absolute extraction efficiencies of the procedure. Fish (rainbow trout) exposed to ^{14}C-EHDP were dissected and individual organs were assayed for EHDP and DPP by gas chromatography. Generally only a small proportion of the radioactivity accumulated by muscle and liver (Table 4) was unextractable using methanol. However, a major portion of the radioactivity was not in the form of DPP or EHDP and was not identified.

DAPs were recovered efficiently from river sediment fortified at 50 and 500 ng/g (Table 5). TPP and EHDP recoveries were somewhat lower than those observed for water samples where a similar volume was passed through the XAD resin column. The lower recovery may be due to traces of methanol in the aqueous phase which may have reduced the extraction efficiency of the resin for nonpolar compounds. Greater than 100% recoveries were calculated for DoCP and TPP at the 50 ng/g level due to interfering GLC peaks of identical retention time. Blank chromatograms for sediment were similar to those for water (Figures 1 and 2).

Recovery of ^{14}C-EHDP and TPP incubated under aerobic and anaerobic (nitrogen atmosphere) conditions with river sediment for 32 and 64 days (Table 6) illustrated that both TAPs and DPP could be extracted but a considerable proportion of the radioactivity from aerobic incubations was unextractable by refluxing with

Table 5. Recovery of diarylphosphates (as butyl esters) and triarylphosphates from fortified river sediment

Compound	Recovery at each concentration (ng/g),[a] %		
	50	500	1000
DPP	92.8 ± 8.5	105.2 ± 15.8	91.6 ± 5.2[b]
DoCP	139.1 ± 26.6	105.6 ± 25.1	—
DmCP	76.3 ± 8.1	94.4 ± 18.4	—
DpCP	82.1 ± 8.8	99.5 ± 27.6	—
TPP	126.4 ± 5.5	75.7 ± 1.8	—
EHDP	110.6 ± 5.3	62.6 ± 7.4	—

[a] Average of 3 replicates ± standard deviation.
[b] Determined by measurement of ^{14}C.

Table 6. Recovery of ^{14}C-TPP and ^{14}C-EHDP incubated with river sediment for 32 and 64 days under aerobic or anaerobic conditions

Time, days	Chemical	Condition	Total extd, %	TPP[a] or EHDP, %	Unextracted, [b] %
32	TPP	aerobic	29.9	69.7	70.1
		anaerobic	59.8	81.6	40.2
	EHDP	aerobic	86.0	87.4	14.0
		anaerobic	97.2	78.7	2.8
64	TPP	aerobic	17.6	46.1	82.4
		anaerobic	22.7	76.7	77.3
	EHDP	aerobic	67.0	70.0	33.0
		anaerobic	91.9	85.3	8.1

[a] Remaining extractable radioactivity mainly in the form of DPP.
[b] Measured by combustion of extracted sediment.

aqueous methanol. The proportion of extractable radioactivity as ^{14}C-DPP in the samples (64 days), resulting from hydrolysis of either TPP or EHDP, ranged from 30 to 54% in aerobic sediments. Whether the unextractable radioactivity was in the form of monophenyl- or diphenylphosphate could not be determined. It might be possible to extract some of this radioactivity with acid or alkaline conditions but, because hydrolysis of TAPs and DPP would be expected, this approach was not investigated.

The results of the analyses of fortified water, fish, and sediment samples indicate that existing procedures for TAPs can be extended to the diaryl degradation products. The major modification required is the use of a partition step between a small volume of acidified aqueous phase and an excess of ethyl acetate to transfer the water-soluble DAPs into the organic phase. Similar procedures have been reported for extraction of dialkylphosphates into the organic phase (9). TPP was also partitioned efficiently with this procedure. EHDP, an alkyldiphenylphosphate, was not recovered efficiently from fish sediment or water, possibly because of losses by volatilization and hydrolysis. As noted by several authors (6, 7, 11), analysis of aryl phosphates is complicated by phosphorus-containing interferences that may originate from laboratory reagents and apparatus. Use of HPLC-grade water instead of glass-distilled and H_2SO_4 instead of concentrated HCl for acidification reduced backgrounds in DAP analysis in the present work.

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Determination of Organic Phosphate Triesters in Human Adipose Tissue

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A method of analysis has been developed for the determination of organic phosphate triesters in human adipose fat at low ng/g levels. After fat extraction from the tissue with benzene (or acetone-hexane, 15 + 85, v/v), phosphates were fractionated from fat by gel permeation chromatography with methylene chloride-cyclohexane (5 + 95, v/v) as solvent. After Florisil column cleanup, the GPC extract was analyzed by capillary column gas chromatography using a nitrogen-phosphorus selective detector. Recoveries at the 2.5, 10, and 25 ng/g levels were greater than 75% except for tri(2,4-xylenyl) phosphate (ca 65%). Of 16 human adipose tissue samples analyzed, 5 contained tris(1,3-dichloropropyl) phosphate in the range of 0.5 to 110 ng/g, 4 contained tributoxyethyl phosphate in the range of 4.0 to 26.8 ng/g, and one contained tributyl phosphate at 9.0 ng/g.

Trialkyl, haloalkyl, and aryl phosphate esters (TAAPs) are widely used as flame retardant plasticizers, fire retardant hydraulic fluids, and additives in lubricants, adhesives, and coatings (1–3). TAAPs have been detected in Canadian potable water supplies (4, 5), fish (6), and laboratory chemicals and equipment (4, 7). Tris(1,3-dichloroisopropyl) phosphate has been detected in human seminal fluid (8).

Several of these compounds have a low solubility in water and a high octanol–water partition coefficient (9), indicating a potential for bioaccumulation in fatty tissues. The bioaccumulation of some TAAPs in fish has been reported by Muir et al. (10, 11) and Lombardo and Egry (6).

Because of the widespread use of TAAPs, their detection in drinking water and fish, and the possibility of their bioaccumulation in human tissue, a method using gel permeation chromatography has been developed for their determination at ng/g levels in human adipose tissue.

METHOD

Reagents

(a) *Solvents.*—Acetone, hexane, cyclohexane, methylene chloride, and benzene (Caledon Laboratories, Georgetown, Ontario): all distilled-in-glass grade. (Caution: Avoid exposure to benzene, a known carcinogen, by handling in fume hood.)

(b) *Glass wool and purified water.*—Prepare as previously described (4).

(c) *Anhydrous sodium sulfate.*—Reagent grade, granular. Wash with methylene chloride. Remove solvent and heat at 400°C overnight. Cool and store in bottle with Teflon-lined cap.

(d) *Standard solutions.*—Prepare 500 ng/μL stock solutions and appropriate working solution mixtures as previously described (4). *o*-Isopropylphenyldiphenyl phosphate (*o*-IPDP) and *p*-*t*-butylphenyldiphenyl phosphate (*p*-*t*-BPDP), 99+% purity, were synthesized by the method of Wightman (12).

(e) *Gel beads.*—Bio-Beads S-X3 (Bio-Rad Laboratories, Mississauga, Ontario), porous styrene-divinyl benzene copolymer, 200–400 mesh.

(f) *Florisil.*—PR grade (Mandel Scientific, Rockport, Ontario). Activate at 275°C overnight, let cool, and deactivate with 2% (w/w) water (previously extracted with hexane). Store in glass jar with Teflon-lined cap.

Note: Do not use foil-lined cardboard-backed caps because these may contain 2-ethylhexyldiphenyl phosphate.

Apparatus

(a) *Manual gel permeation chromatography (GPC) apparatus.*—High pressure metering pump (Eldex Model 8-100-S); sample injection valve (Rheodyne Model 5020); sample loading loop consisting of appropriate length of 1/16 in. Teflon tubing with Cheminert unions; pulse dampener consisting of 6 ft × 1/8 in. copper tubing coiled, closed at one end, and installed in-line between pump and sample injection valve. Place low pressure, 2 μm solvent filter at inlet of pump intake line.

(b) *GPC column.*—2.5 cm id × 45 cm column (Pharmacia Model SR 25/45) fitted with organic solvent-resistant plungers.

(c) *Extraction apparatus.*—Tekmar SDT series overhead Tissumizer with SDT-182 EN shaft and generator for use in water and/or organic solvent medium.

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(d) *Centrifuge.*—Table top, IEC Model HNSII. Operate at 1600 rpm.

(e) *Chromatography column.*—Chromaflex column, 6 mm id, with 50 mL solvent reservoir (Kontes No. K-420100-0021).

(f) *Gas chromatographs.*—

(*i*) Perkin Elmer Model 910 equipped with nitrogen–phosphorus selective detector (NPD). Column parameters and operating conditions: 30 m × 0.25 mm id DB-1 (J & W) fused silica capillary column. Using syringe (Hamilton, Model 701SN) with 3 in. needle, introduce aliquot through splitless injector system (SGE) at 70°C, open vent at 20 s, and at 60 s increase initial temperature to 170°C, hold 2 min, program at 8°/min to 260°C, hold 5 min, post-program temperature 4 min at 265°C. Helium carrier gas velocity of 45 cm/s at 25 psig with make-up flow of 25 mL/min; injector 270°C; detector interface 270°C. Adjust detector bead temperature setting for full scale deflection with 50 pg triphenyl phosphate.

(*ii*) Varian Vista 44 equipped with flame photometric detector (FPD) operating in the phosphorus mode. Column parameters and operating conditions: 30 m × 0.32 mm id DB-5 (J & W) fused silica capillary column; oven temperature: initial 50°C, hold 1 min, program at 40°/min to 180°C (hold 1 min), then program 5°/min to 250°C (hold 1 min); post-program temperature 5 min at 260°C; nitrogen carrier gas velocity ca 35 cm/s with make-up gas flow of 30 mL/min. Detector gas flows: hydrogen, 140 mL/min; air No. 1, 80 mL/min; air No. 2, 180 mL/min.

(g) *Gas chromatograph–mass spectrometer (GC/MS).*—Finnigan Model 4000, coupled with series 6110 data system and containing 30 m × 0.25 mm id DB-5 (J & W) fused silica capillary column. Operating conditions: temperature 50°C for 0.1 min, program at 15°/min to 150°C, then at 5°/min to 250°C; ion source 250°C.

Extraction of Human Adipose Tissue

Let deep-frozen (−20°C) tissue, obtained from cadavers at autopsy and stored in clean vials, thaw overnight in cold room (4°C) and bring to room temperature ca 30 min before extraction. Cut small portions of tissue on piece of acetone-rinsed heavy aluminum foil. Accurately weigh ca 3 g tissue into 50 mL round-bottom centrifuge tube with pouring spout. Add 15 mL benzene or acetone–hexane (15 + 85, v/v) solvent mixture and ca 1 g precleaned anhydrous sodium sulfate. Homogenize mixture with Tissumizer® at moderate speed for ca 2 min. (Note: Connective tissues may block homogenizer blades; carefully control speed to avoid splashing.) Rinse homogenizer shaft with ca 20 mL solvent and collect all rinsings. Centrifuge extract at 1600 rpm ca 15 min, and filter clear solution through anhydrous sodium sulfate. Rinse twice with 5 mL solvent and concentrate to dryness using rotary evaporator. Transfer adipose fat to graduated centrifuge tube with methylene chloride–cyclohexane (5 + 95, v/v) solution (GPC solvent) and dilute to 12 mL. Transfer 1 mL aliquot to pre-weighed 0.5 dram vial, evaporate solvent with stream of nitrogen, and determine fat concentration by weighing.

Gel Permeation Chromatography (GPC)

(a) *Preparation of column.*—Slurry 50 g Bio-Beads S-X3 in methylene chloride–cyclohexane (5 + 95, v/v) and pack GPC column. Compress gel to a bed height of 10.5 in. (26.7 cm) (130 mL). Equilibrate system by running GPC solvent mixture 10–15 min before sample injection. Flush sample loop with solvent mixture before loading loop.

(b) *Calibration of GPC column.*—Dissolve extracted fat in GPC solvent mixture at concentration of 0.2 g/mL or prepare standard mixture solution in GPC solvent mixture. Load sample loop with appropriate solution and inject onto GPC column. For fat calibration, collect 10 mL fractions in graduated centrifuge tubes, evaporate to ca 0.5 mL on a N-Evap® multi-port evaporator, and quantitatively transfer residue to preweighed 0.5 dram vials. Evaporate solvent to a constant weight extract and determine elution profile. For TAAP-GPC calibration, collect 10 mL (or 15 mL) fractions, evaporate to ca 0.5 mL (to displace any methylene chloride which might adversely affect NP detector) and dilute to appropriate volume with acetone for GC/NPD analysis. Determine TAAP-GPC elution profile.

(c) *GPC fractionation of fat solution.*—Using syringe previously rinsed with small amount of fat solution (ca 0.2 mL), load calibrated 5 mL sample loop with fat solution (≤1.25 g total fat). Transfer sample from loop onto GPC column and elute with solvent mixture at 5 mL/min. Discard first 85 mL and collect Fraction I (85–115 mL) containing tributyl and tributoxyethyl phosphate and small amount of fat; and Fraction II (115–330 mL) containing the remaining TAAPs. Evaporate the 2 fractions to ca 1–2 mL, using rotary evaporator, transfer to calibrated centrifuge tube with hexane, and concentrate to 0.3 mL,

Table 1. Comparison of fat cleanup methods

Parameter	Extraction				
	Acetonitrile-hexane (butter)				GPC (adipose)
Weight of sample, g	2.50		3.10		1.0
After cleanup, g	0.152		0.183		ca 1–4 mg
Cleanup efficiency, %	94.0		94.2		99.6–99.9
Column cleanup:	Florisil[a]	Florisil[a]	Florisil[b]	Alumina[c]	H_2SO_4–alumina[d]
Weight fat applied, mg	152	183	13.3	13.3	13.3
After cleanup,[e] mg	140	171	13.3	13.3	13.2

[a] 15 g Florisil column.
[b] 1 g mini-Florisil column.
[c] 1 g alumina column.
[d] 1 g H_2SO_4–alumina column.
[e] Fraction containing TAAPs.

using gentle stream of nitrogen, for GC analysis or further Florisil column cleanup.

Florisil Column Cleanup

Fraction I.—Insert small glass wool plug in 0.6 cm id Chromaflex column, pack with 1 g 2% deactivated Florisil, and top with ca 5 mm anhydrous sodium sulfate. Apply Fraction I extract (0.3 mL) with solvent-rinsed Pasteur pipet to column followed by 0.5 mL hexane rinse of tube as soon as extract reaches top of column. Elute with 5 mL acetone–hexane (5 + 95, v/v) solution followed by 3 mL acetone–hexane (50 + 50, v/v) solution. Discard 0–5 mL fraction containing fat and collect 5–7.5 mL fraction containing tributyl phosphate and tributoxyethyl phosphate. Concentrate eluate to 0.2–0.5 mL with gentle stream of nitrogen for GC/NPD analysis.

Fraction II.—Prepare column as for Fraction I but use 2 g 2% deactivated Florisil topped with ca 5 mm anhydrous sodium sulfate. Wash Florisil column with ca 10 mL hexane and, as hexane reaches top of adsorbent, add Fraction II concentrate (0.3 mL). Rinse extract tube with 0.2 mL hexane and immediately add rinse to column as layer reaches adsorbent. Elute with 4.5 mL hexane (5 mL total hexane), 5 mL acetone–hexane (5 + 95, v/v) solution, and then 8 mL acetone–hexane (50 + 50, v/v) solution. Collect fractions in graduated 5 mL centrifuge tubes as follows: Discard holdup volume as predetermined (ca 0–4 mL); Fraction A (4–8 mL) containing PCBs, DDE, and HCB; Fraction B (8–12 mL) containing DDT and several other organochlorine pesticides; and Fraction C (12–17 mL) containing organic phosphate triesters. Concentrate Fraction C to 0.2–0.5 mL under gentle stream of dry nitrogen for GC analysis.

TAAP elution volumes for the Florisil cleanup should be predetermined by calibration using appropriate TAAP.

Recovery Studies

Before homogenization, fortify mixture of tissue (or GPC-cleaned fat) and extracting solvent in centrifuge tube with known amounts of standards mixture solution to give fortification levels of 2.5, 10, and 25 ng/g, on whole-tissue basis. Proceed as previously described for extraction of adipose fat from tissue and for GPC cleanup.

Quantitation by GC and Confirmation by GC/MS

Proceed as previously described (4).

Results and Discussion

Attempts to isolate TAAPs from human adipose tissue by using the standard residue procedure (13) were unsuccessful due to the relatively large amount of fat co-extracted by the solvent, the incomplete extraction of the more polar phosphates, and the co-elution of the co-extracted fat with TAAPs during Florisil column cleanup (Table 1). However, gel permeation chromatography with Bio-Beads S-X3 has been used to isolate organophosphate, triazine, and carbamate pesticides from fat samples (14) and to isolate triaryl phosphates from fish (15). This GPC technique was therefore evaluated for the separation of TAAPs from human adipose fat with methylene chloride (5, 10, or 15%) in cyclohexane as the solvent. The best separation of fat/phosphates was obtained with the lower (5%) concentration of methylene chloride but at the expense of higher elution volumes for the late-

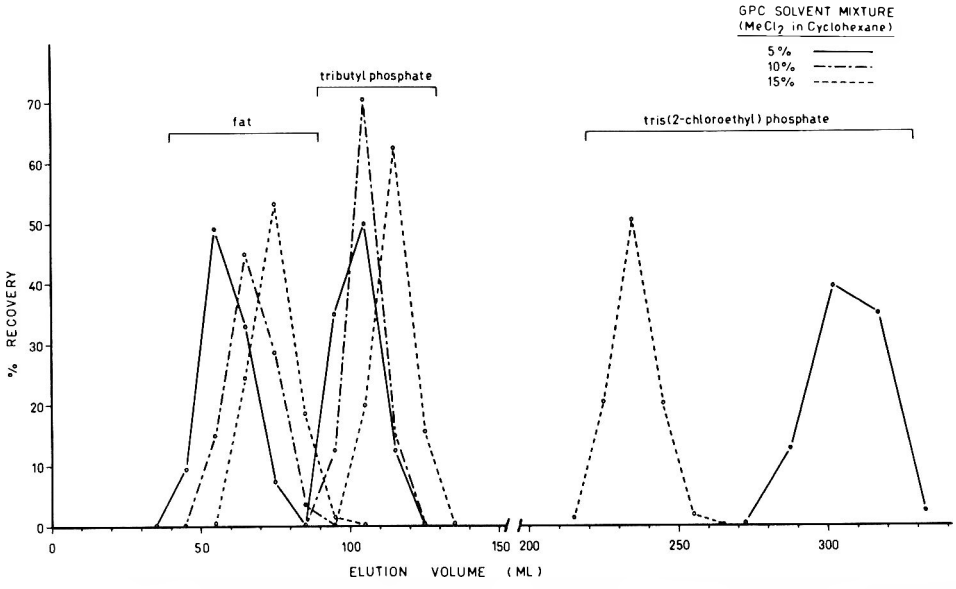

Figure 1. GPC elution profiles of adipose fat, tributyl phosphate, and tris(2-chloroethyl) phosphate with various eluant mixtures.

eluting phosphates (Figure 1). The gel bed compactness also affected the efficiency of the separation (Figure 2); the more compressed the packing the better the resolution, but pressure limitations of the system also had to be considered.

The selected system for all further studies consisted of a GPC column packed with 50 g Bio-Beads S-X3 gel compressed to a bed volume of 130 mL (10.5 in.) using methylene chloride–cyclohexane (5 + 95, v/v) elution solvent. Using this system, most TAAPs could be separated (Fraction II, 115–330 mL cut) from adipose tissue to produce extracts clean enough for direct analysis on GC-NPD with a capillary column. From a 1 g sample injected on the GPC column, only about 0.1 mg fat remained in the extract for 99.99% cleanup efficiency.

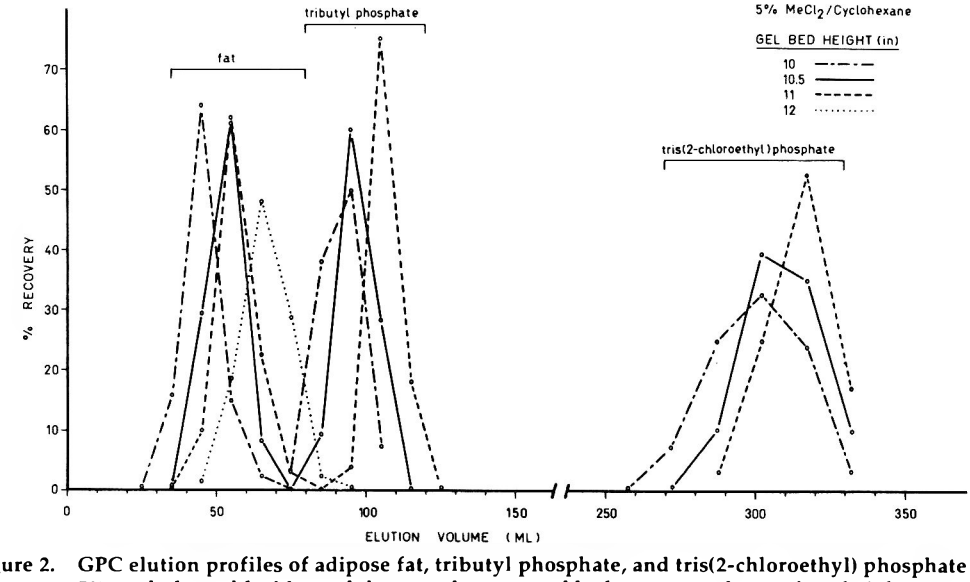

Figure 2. GPC elution profiles of adipose fat, tributyl phosphate, and tris(2-chloroethyl) phosphate with 5% methylene chloride-cyclohexane eluant on gel bed compressed to various heights.

Table 2. GPC, GC, and GC/MS data for some organic phosphates

Phosphate	GPC elution vols,[a] mL	GC retention time,[b] s	GC/MS Selected Ions	
Tri(2-ethylhexyl)	50–80			
Tributyl	85–115	373	155	211
Tributoxyethyl	85–115	856	125	299
Tri(2,4-xylenyl)	110–150	1280	305	410
p-*t*-Butylphenyldiphenyl	120–160	1100	367	382
o-Isopropylphenyldiphenyl	120–175	935	251	368
Tri-*o*-tolyl	120–175	976	91	368
Tri-*m*-tolyl	130–190	1019	91	368
Triphenyl	160–220	838	77	326
2-Ethylhexyldiphenyl		868	251	362
Tris(1,3-dichloroisopropyl)	190–250	796	191	381
Tris(2,3-dichloro-*n*-propyl)		824	191	381
Tris(2-chloroethyl)	265–340	422	143	249

[a] 130 mL bed volume S-X3 gel with methylene chloride–cyclohexane (5 + 95, v/v) eluant.
[b] DB-1 column.

The elution volumes of TAAPs (Table 2), in addition to the expected size dependence within a class, appeared to depend on the type of phosphates, i.e., trialkyl < triaryl < tri(haloalkyl). Tris(2-ethylhexyl) phosphate, the largest of the trialkyl phosphates studied, could not be adequately separated from the fat with any of the systems used; therefore, no further attempts were made to include this compound in the study.

Tributyl phosphate (TBP) and tributoxyethyl phosphate (TBEP) are also not eluted in the 115–330 mL fraction, and a separate fraction (Fraction I, 85–115 mL) was collected to isolate these 2 compounds. This fraction contained 1 to 4 mg residual fat, which adversely affected the resolution and lifetime of the capillary GC columns used for analysis, and a further cleanup step was required. Alumina (15) or sulfuric acid-impregnated alumina (16) column chromatography was not satisfactory, but TBP and TBEP, unlike the triaryl phosphates, could be easily separated from the fat by using a Florisil column (Table 3).

Preliminary analysis of human adipose fat samples revealed several peaks on the GC/NPD chromatogram for Fraction II, most particularly when applied for detection in the 0.5–5 ng/g range (Figure 3a). These were attributed partly to organochlorine pesticides (i.e., DDE) and PCBs known to be present in these human adipose tissues at the μg/g level. The NP detector will respond to high levels of these compounds and, to simplify GC identification and quantitation and facilitate GC/MS confirmation, Fraction II was further fractionated by Florisil column chromatography.

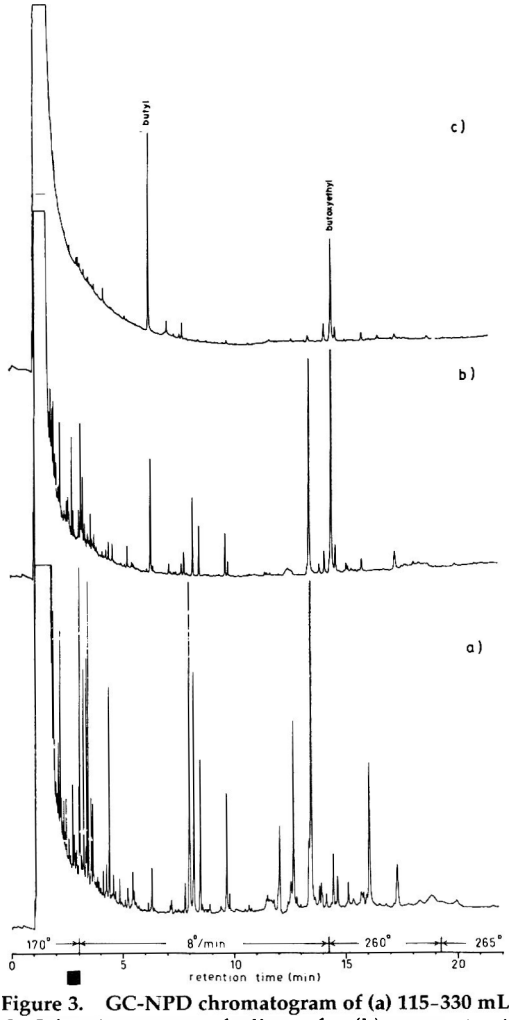

Figure 3. GC-NPD chromatogram of (a) 115–330 mL GPC fraction extract of adipose fat; (b) same extract further cleaned by Florisil; (c) system blank.

Table 3. Mini-Florisil column calibration [a]

Elution vol., mL	Fat (13.3 mg applied), % eluted	Phosphate triesters									
		n-Butyl	2-Cl.ethyl	1,3-Dichl.pro	Phenyl	Butoxyethyl	*o*-IPDP	*o*-Tolyl	*m*-Tolyl	*p*-*t*-But.PDP	2,4-Xylyl
0–1	—	—	—	—	—	—	—	—	—	—	—
1–2	9.4	—	—	—	—	—	—	—	—	—	—
2–3	91.6	—	—	—	—	—	3.1	11.9	2.2	2.6	29.7
3–4	<0.1	—	—	—	1.3	—	29.5	48.6	26.0	25.6	52.6
4–5	—	—	—	—	9.1	—	40.0	30.9	39.2	38.4	15.3
5–7.5	—	75.2	—	17.7	89.6	—	27.4	8.6	32.7	33.4	3.4
7.5–10	—	24.8	—	82.3	—	2.2	—	—	—	—	—
10–12.5	—	—	—	—	—	63.2	—	—	—	—	—
12.5–15	—	—	3.4	—	—	25.8	—	—	—	—	—
15–20	—	—	96.6	—	—	9.8	—	—	—	—	—

[a] Sample applied as 1 mL solution in hexane, and eluted with 5 mL acetone–hexane (5 + 95, v/v) followed by acetone–hexane (15 + 85, v/v) solution.

Figure 3b shows a chromatogram of a Florisil-cleaned GPC extract. This illustrates a much simpler chromatogram compared with the main GPC extract before Florisil cleanup. However, several other unidentified peaks appear in the GC/NPD chromatogram of several extracts, but their identification was not possible because amounts were insufficient for MS analysis.

Recovery studies using the fully developed method were carried out at 2.5, 10, and 25 ng/g fortification levels using benzene as extracting solvent. Unfortified fat from the same sample was processed through the method to determine the blank contribution. To eliminate possible TAAP contribution from the adipose fat, GPC processed fat (waste fractions from previous GPC runs) was used for spiking purposes at the 2.5 ng/g fortification level. Results are shown in Table 4 and have been corrected for sample blank contribution. The low tributoxyethyl phosphate recovery at 10 ng/g could not be explained although preliminary studies using butter as substrate gave 75% recovery.

Extraction of fat from the adipose tissue with benzene followed a method for determination of PCBs in blood (17). However, because of concerns on the health hazard associated with benzene, acetone–hexane (15 + 85, v/v) was also used as an alternative extracting solvent. Analysis of spiked fat extracted with acetone–hexane mixture produced results similar to benzene-extracted spiked fat.

GC/NPD detection limits based on a 1 cm peak for a 1 g sample extract concentrated to 0.2 mL (2 μL injected) were less than 1 ng/g. However, because of the presence of TBP and TBEP in laboratory air, it was impossible to avoid their presence in the system blanks (Figure 3c), and the practical method detection limits for these 2 phosphates were set at ca 2 ng/g (2× blank levels).

Sixteen samples of human adipose tissue were analyzed for TAAPs included in the study. The results (Table 5) have been calculated on an extracted fat basis. The amount of extracted fat from the tissue samples ranged from 74.9 to 89.6%. No triaryl or trialkyl/aryl phosphates were found in any of the samples. Tris(1,3-dichloroisopropyl) phosphate was found in 5 samples at concentrations ranging from 0.5 to 110 ng/g. The sample containing a high level of tris(1,3-dichloropropyl) phosphate had an unknown peak (Figure 4b) following the dichloropropyl phosphate and this has been identified by GC/MS as a dichloropropyl phosphate isomer. However, since no tris(2,3-dichloro-*n*-

Table 4. Recoveries of trialkyl/aryl phosphates

Phosphate	Human adipose fat,[a,b] benzene extraction		
	2.5 ng/g	10 ng/g	25 ng/g
Tributyl	77.7 ± 12.5	90.6 ± 14.5	108.4 ± 13.7
Tributoxyethyl	78.2 ± 6.8	52.3 ± 5.0	87.7 ± 9.1
Tris(2-chloroethyl)	82.8 ± 9.5	98.8 ± 11.9	94.5 ± 13.6
Tris(1,3-dichloropropyl)	92.1 ± 11.2	98.5 ± 13.3	111.4 ± 8.4
Triphenyl	95.9 ± 10.2	99.8 ± 14.0	104.9 ± 3.8
Tri(o-tolyl)	91.1 ± 9.4	100.8 ± 14.7	105.4 ± 4.8
Tri(*m*-tolyl)	88.7 ± 11.4	96.3 ± 9.7	105.9 ± 4.1
Tri(2,4-xylenyl)	63.7 ± 10.4	67.8 ± 11.5	68.6 ± 8.9
o-Isopropylphenyldiphenyl	93.9 ± 13.2	97.1 ± 17.4	105.1 ± 3.6
p-t-Butylphenyldiphenyl	90.7 ± 12.5	96.7 ± 17.5	93.4 ± 8.8

[a] GC/NPD analysis of Fractions I and II.
[b] % recovery ± SD; triplicate determinations.

propyl) phosphate standard could be obtained [one source of tris(2,3-dichloropropyl) phosphate was found to be the 1,3-isopropyl isomer by NMR], its concentration was estimated at 4.6 ng/g by comparison with the 1,3-isopropyl isomer standard and assuming a similar NPD response. Tributoxyethyl phosphate was detected in 4 samples, and tributyl phosphate was found in one sample at significant levels above the blank value. All results are obtained for GC/NPD analysis on a DB-1 capillary column; however, identity was confirmed by GC/MS by searching reconstructed ion chromatograms for at least 2 characteristic ions for the sample and standards within retention time windows established for the standards. Confirmation by GC/MS required analysis of an extract aliquot containing ca 1 ng suspected phosphate (Table 5).

Phosphates below 10 ng/g were also confirmed, after concentration of the extract to 10 μL, by GC analysis of a 2 μL aliquot using a DB-5 capillary column and a flame photometric detector.

In conclusion, a GPC method has been developed for the determination of trialkyl, trihaloalkyl, and trialkyl/aryl phosphates in human adipose fat at the ng/g level. However, the application of the method to these low concentrations requires careful manipulation to avoid contamination from laboratory products and chemicals. The method promises the possibility of establishing a multi-class organic screening method; similar systems have been reported for the analysis of organochlorine pesticides and PCBs in human adipose tissue (18).

Acknowledgment

We thank Frank Benoit and Ron O'Grady for GC/MS information, Sharon Murray for tech-

Table 5. TAAP concentration in human adipose tissue

Sample	Extd fat, %	TAAPs, ng/g[a]			
		n-Butyl	Butoxyethyl	1,3-Dichloropropyl	Others
1	79.4	—	—	1.6[b]	—
2	85.7	—	—	9.2[b,c]	—
3	81.9	—	26.8[c]	0.6[b]	—
4	89.5	—	—	0.5[b]	—
5	75.8	9.0[b]	4.9[b]	—	—
6	89.3	—	—	110[b,c]	dichloropropyl isomer, 4.6[c]
7	89.6	—	4.0[b]	—	—
8	74.9	—	7.4[b]	—	—
9–16	75.2–85.1	ND[d]	ND	ND	ND

[a] Calculated on extracted fat basis.
[b] Confirmation by GC-FPD with DB-5 column.
[c] Confirmation by GC/MS.
[d] ND = not detected.

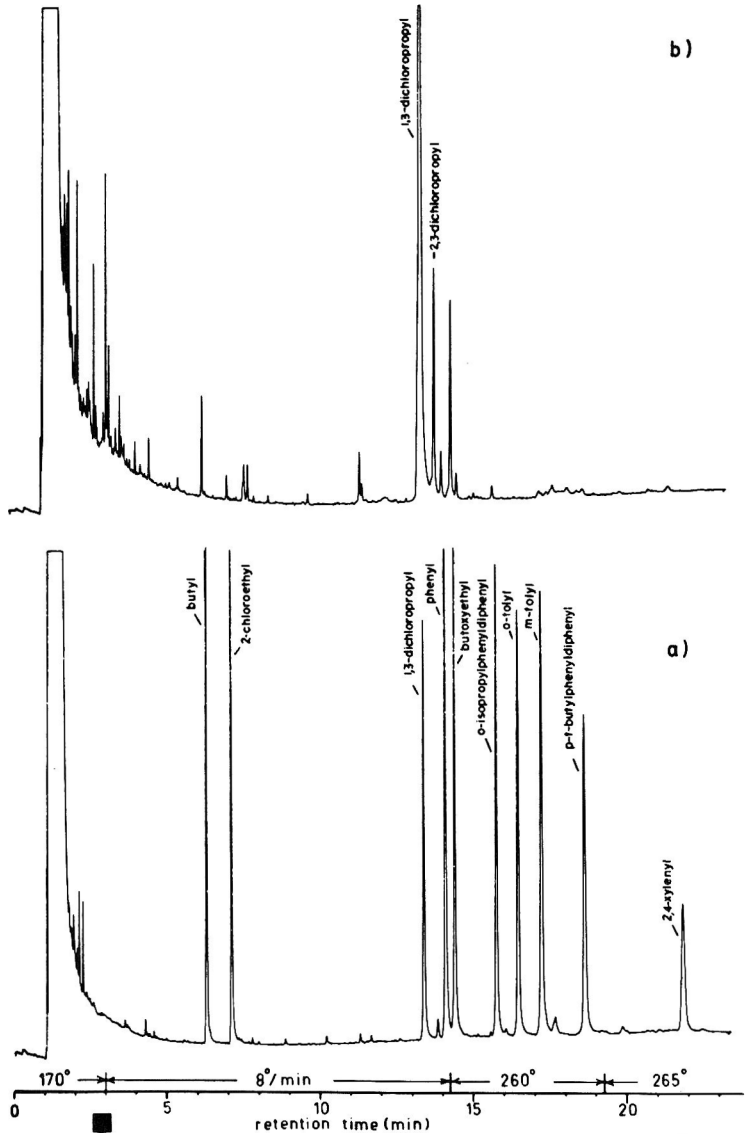

Figure 4. GC-NPD chromatogram of (a) 50 pg each of TAAP standard mixture; (b) adipose fat extract containing tris(dichloropropyl) phosphate.

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Interlaboratory Study on Determination of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Fish

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An interlaboratory round robin study was carried out to estimate the reliability of data on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in fish. Using different methods, 13 laboratories (4 Canadian, 9 American) agreed to analyze 4 fish samples; 3 were Great Lakes salmonids containing bio-incurred levels of TCDD below 100 ppt and the fourth was an ocean fish fillet containing no measurable TCDD. Samples were sent as freeze-dried portions as it was shown that no change of TCDD occurred by this sample preparation. Results were normalized between laboratories by supplying each with an aliquot of the same 2,3,7,8-TCDD standard. Eight laboratories reported a set of results of which one set was rejected. Values from the 7 remaining laboratories for the 3 positive fish showed mean concentrations in pg/g (ppt) and (CV, %) of 61.2 (13.9), 30.4 (18.4), and 32.3 (25.4). Detection limits averaged 3.6 ppt and ranged between 1 and 10 ppt. No significant differences appeared in the concentration of 2,3,7,8-TCDD in fish samples from methods differing in the use of: (i) digestion or extraction techniques, (ii) high or low resolution mass spectrometry, and (iii) isomer specific or nonspecific separations. Overall recovery values using internal standards varied greatly (29-109%) even within the same laboratory and pointed to the need to use an internal standard to obtain precise results. Agreement among laboratories was good considering the level quantitated (ppt) and the diverse methodology.

In late 1978, the U.S. Environmental Protection Agency (EPA) (1, 2) reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was present in fish from waters flowing into Lake Huron in the vicinity of Saginaw Bay. In early 1979, the New York State Department of Health released preliminary data (3) showing levels of the same compound in a limited number of samples of Lake Ontario fish. At about the same time, analysis of commercial fish by the Canadian Health Protection Branch (HPB) indicated that TCDD residues were present but the status of the methodology was uncertain. Again in 1980, the Canadian Wildlife Service found 2,3,7,8-TCDD in herring gull eggs (a fish-eating species) from the Great Lakes with highest concentrations in colonies from Lakes Ontario and Huron (4). Subsequent to these findings an international meeting of scientific and political advisors was convened in Washington to consider the implications of 2,3,7,8-TCDD in Great Lakes biota. One of the first questions that arose was the reliability of the data, in particular, the reliability of the methods used to generate the residue data on 2,3,7,8-TCDD in fish. As a result, the 2 governments initiated an exchange of Great Lakes fish samples to those laboratories capable of analyzing fish for low (parts per trillion, 10^{-12}; ppt) levels of 2,3,7,8-TCDD. Information was sought on the variation both within and between laboratories, precision, accuracy, specificity, detection limits of various methods, and sample turnover time.

A number of method comparisons and validations have been carried out with chlorinated dibenzo-*p*-dioxins. An earlier report by Villanueva et al. (5) compared 4 analytical methods for dioxins in pentachlorophenol preparations and found a wide variation in both the levels, number of steps, and recoveries using gas chromatography (GC) with electron capture (EC) detection. A method validation study (6) of TCDD in spiked standard solutions and beef fat tissue using EPA acid cleanup method followed by GC–high resolution mass spectrometry (MS) separation and detection has been carried out. In this study from 2 laboratories, TCDD could be statistically quantitated in some beef fat as low as 9 ppt although detection limits varied from 3 to 18 ppt. More recently, a study (7) of various techniques for cleanup of 2,3,7,8-TCDD in fish samples was carried out by the U.S. Food and Drug Administration (FDA). Six samples of fortified and unfortified fish were cleaned up by 6 separate laboratories using their methodology, and TCDD in the extracts was determined by a single laboratory (FDA) using capillary GC with multiple ion MS detection. The 6 laboratories were ranked according to the degree of interferences and amount of co-extractives in the final

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Table 1. History and properties of fish samples sent in freeze-dried portions to participating laboratories

Property	Sample A	Sample B	Sample C	Sample D
Species	lake trout	ocean haddock	lake trout	rainbow trout
Portion	whole fish	fillet	whole fish	whole fish
Collection location	Burlington, Lake Ontario	East Coast Canada	Burnt Island, Lake Huron	Toronto harbor, Lake Ontario
Collection date	9-16-80	5-4-81	8-15-80	8-20-80
Lipid content,[a] %	19.8	0.4	24.7	18.5
Weight (g) of freeze-dried aliquot (= 50 g wet tissue)	21.4	10.0	20.6	18.6
PCB,[b] ppm	7.28	ND[c] (0.01)	5.03	1.77

[a] Determined by method of Schmitt et al. (8).
[b] Reported as total of Aroclors 1242, 1254, and 1260; analysis carried out by FWS.
[c] Not detected, followed by, in parentheses, detection limit in μg/g (ppm).

extract. The 6 laboratories as judged by the FDA MS fell into 4 categories varying from no interferences or co-extractives to large amounts of both.

In the present study, 13 laboratories (4 Canadian, 9 American) having experience in the determination of low levels (ppt) of 2,3,7,8-TCDD in biological samples agreed to analyze 4 fish samples, using their extraction, cleanup, and detection procedures, and to report their results. Eight of the 13 laboratories successfully completed the study in the allotted time. Their results are the subject of this report.

Experimental

Interlaboratory Protocol

Each laboratory received 4 samples of freeze-dried fish, 3 of which contained bio-incurred levels of 2,3,7,8-TCDD. They were requested to analyze the fish samples with the provided standard using their own laboratory method and to report the results. Laboratory protocol similarities were measurement technique and use of the 4 fish samples and 2,3,7,8-TCDD standard that were provided. Differences involved extraction and cleanup procedures and MS instrumentation. The laboratories (4 Canadian, 9 American) and the main investigators are listed in *Acknowledgments.*

Fish Samples

The history and properties of the fish used in this study are given in Table 1. Three of the samples were whole fish salmonids from the Great Lakes and had a high lipid content and high levels of PCB. The fourth fish, sample B, was an ocean fillet of low lipid and PCB content used as a blank sample. Lipids were determined by the method of Schmitt et al. (8) and PCB as the sum of Aroclors 1242, 1254, and 1260 by the U.S. Fish and Wildlife Service (FWS). Between 1.0 and 1.5 kg wet fish sample was homogenized in a tissue grinder and 100–200 g aliquots were lyophilized in a Virtis Model No. 10-030 freeze-dryer. The powdery, somewhat oily material, was mixed and pulverized in a Waring blender and the entire sample was rehomogenized in a Hobart food mixer equipped with a wire beater/whipper. Aliquots equivalent to 50 g wet tissue (actual weights in Table 1) of each fish sample were packaged in toluene–acetone washed 4 oz bottles and shipped to the laboratories by either postal or courier service. Each package contained a set of instructions outlining the purpose of the study, the nature of the samples, the information desired from each laboratory, and a time limit on reporting results. Each laboratory was asked to submit (a) a brief description of the method used including the specificity and type of MS instrument; (b) an estimate of the detection limit of 2,3,7,8-TCDD both alone and in the fish sample; (c) copies of pertinent chromatograms; and (d) concentration of 2,3,7,8-TCDD in the 4 samples.

2,3,7,8-TCDD Standard

To normalize the standards between laboratories such that the variation in results was due to the method alone, each participating laboratory received from FDA, Washington, DC, at the same time fish samples were sent, a sealed glass ampule containing 5 mL 2,3,7,8-TCDD in isooctane at a concentration of 1.16 μg/mL. Concentration was verified at FDA by UV spectrometry. This solution was to be used by each laboratory either directly or after dilution for determining TCDD levels in the fish.

Evaluation of Effects of Freeze-Drying on TCDD Levels

Radioisotope-labeled 2,3,7,8-TCDD was available from KOR Isotope, Cambridge, MA. One form was ring-labeled ^{14}C-2,3,7,8-TCDD of specific activity 185 mCi/mmole and 99+% chemical purity as determined by GC–EC on a 1% OV-17 glass column, 120 mm × 4 mm id, at 210°C. The other isotopic form was ^{3}H-labeled of specific activity 40 Ci/mmole. Chemical purity of the ^{3}H-2,3,7,8-TCDD as determined above by GC–EC was ca 60% with 25–30% of a possible pentachlorodioxin congener present. Elution of an aliquot on a reverse phase HPLC C18 column, 250 mm × 4.6 mm id, with 1.0 mL/min of 100% methanol followed by fraction collection and liquid scintillation counting in a Beckman LS-230 counter gave a radioisotope purity for 2,3,7,8-TCDD of 48%. Smelt samples (10.0 g aliquots) were fortified in 1.0 mL methanol with radioactive 2,3,7,8-TCDD at 15 and 43 pg/g fish for ^{3}H- (not purified) and ^{14}C-TCDD, respectively. One set of samples was lyophilized at −60°C and 0.1–0.03 mm Hg pressure, an equivalent amount of water was added back to the powdery material, and the samples were vortex-mixed. This set plus a second set which was fortified with 2,3,7,8-TCDD at the same level but not lyophilized were then extracted with chloroform–methanol by the method used at HPB, laboratory 3 (see below). Chloroform extracts were concentrated to near dryness and the residue was taken up in 1.0 mL hexane. Radioactivity in these extracts was then counted with correction for quenching using an external standard.

A second check on the freeze-drying procedure was made by complete sample analysis. 2,3,7,8-TCDD was determined in 3 of the samples (A, C, D) by the method of HPB both with and without freeze-drying, and the values were compared.

Methodology

Methods used by the 8 reporting laboratories are summarized in Table 2 (extraction and cleanup) and Table 3 (MS determination).

Results and Discussion

Effect of Freeze-Drying on TCDD Levels

Freeze-drying was chosen as the method of sample preparation because of the ease with which samples can be homogenized and aliquots can be stored and shipped to various locations. This is particularly important when samples must pass through customs inspection and are thus liable to be held up in transit. Table 4 summarizes the effect of freeze-drying on TCDD levels for both radioisotope (fortified) and entire method (bio-incurred) samples. For the radioisotope experiment, there was no difference in recovery of radioactivity between the 2 treatments. The higher recoveries for carbon-14 than for hydrogen-3 probably reflect the higher purity of the former standard. For the bio-incurred samples analyzed by the HPB method, again no systematic difference in the level of 2,3,7,8-TCDD was noted between freeze-drying and non-freeze-drying. It was concluded that freeze-drying had little or no effect on either fortified or bio-incurred levels of 2,3,7,8-TCDD in fish. Hill and Smart (9) have shown that dehydrochlorination of some organochlorines (hexachlorocyclohexane isomers and DDT) can occur in freeze-dried egg samples, and Coburn and Whittle of Canada Centre for Inland Waters (personal communication) have indicated that hexachlorobenzene levels decrease in fish on freeze-drying. These losses could be due to the higher volatility of the compounds cited or to an increased binding to sample constituents. In any case, these complications do not appear to arise with 2,3,7,8-TCDD in fish samples.

Methodology

The methods used by the 8 reporting laboratories (Tables 2 and 3) can be summarized as follows: Five used solvent extraction of sample and 3 used digestion with either acid or base followed by solvent extraction. Seven of the laboratories used gravity flow chromatographic columns on various adsorbents and 4 of 8 laboratories used reverse phase HPLC as both a separation and cleanup technique. Sulfuric acid, either as a liquid or adsorbed on an inert support, was used by all but one laboratory to degrade lipid material and partition it from the organic phase. All but 2 laboratories used an isotope-labeled stable form of 2,3,7,8-TCDD (either carbon-13 or chlorine-37) as an internal standard at levels between 20 and 1000 pg/g.

MS with electron impact ionization coupled to GC and capillary columns (either fused silica or wall coated glass) was the measurement technique used by all reporting laboratories. The mass spectrometers were either low resolution (5 labs), both magnetic and quadrupole, or high resolution (3 labs) instruments with detection limits for a standard varying between 0.1 and 40 pg. This meant that varying amounts (0.5

Table 2. Summary of cleanup and extraction techniques for determination of 2,3,7,8-TCDD in fish

Lab. No.	Cleanup and extraction	Internal standard recoveries	Remarks
1	20 g fish, duplicate samples (one fortified at 60 ppt for recovery): digest with 20% KOH, room temp., extract with hexane, wash with conc. H_2SO_4; HPLC (3 columns): (a) size exclusion, (b) reverse phase-C8, (c) reverse phase-C18	duplicate samples (one fortified, one unfortified) (no internal standard); ^{13}C-TCDD used for MS only	reagent blank carried through each set of samples
3	10 g fish, blend-extract with $CHCl_3$, methanol, water (Bligh-Dyer); exchange with hexane, wash with conc. H_2SO_4; Florisil minicolumn, HPLC reverse phase, elute with methanol, collect 4–5 mL	^{13}C-2,3,7,8-TCDD 0.5 ng ≡ 50 ppt	reagent blank or spike carried through each set of samples
4	10 g fish (1) acid–base method KOH digestion under reflux, extract with hexane, wash with conc. H_2SO_4, 2 alumina adsorption columns (2) neutral method acetonitrile extraction, partition with hexane, then CH_3CN, Florisil column, then alumina column	^{37}Cl-TCDD 10 ng ≡ 1 ppb	results for 2 methods given and preference stated for neutral method which was reported; chromatograms contain multiple peaks even at 8000 resolution; no other TCDDs detected but TCDF present; quality assurance program
5	50 g fish, extract into hexane; series of silica adsorbents (potassium silicate, silica gel, cesium silicate), then charcoal on urethane foam, reverse elution with toluene, 3rd column of cesium silicate and H_2SO_4 on silica gel, 4th column of alumina	^{13}C-TCDD 5.0 ng ≡ 100 ppt	samples A and D gave extraction problems on charcoal foam system due to polymerized material; needed 50 g sample for single analysis due to low MS sensitivity
6	50 g fish; add water, add Na_2SO_4, extract in column with hexane; Florisil column, elute with 5% water–CH_3CN, partition with hexane–CH_2Cl_2; Florisil column, elute with ether–hexane, defat with H_2SO_4 on silica gel; alumina column, elute with 20% CH_2Cl_2–hexane	no internal standard (recoveries of TCDD from other fish samples at 20 ppt level, 67 ± 13%)	problems with analysis of freeze-dried samples (emulsion); ion ratios given for 2 positive results which were also confirmed on Varian Mat 311A at res. 8000
7	17–25 g fish, freeze-dried; extract with dichloromethane, exchange with hexane; adsorption columns: MgO–Celite, alumina-reverse flow, Florisil-alumina; HPLC: (a) reverse phase-C18-methanol, (b) silica-0.4% toluene in hexane	^{37}Cl-TCDD 780 pg ≡ 31.2 ppt	MS scans region rather than peak maximum; ratios of ions also reported; recoveries low but results compare well with average
9	25 g fish, add water, powder with Na_2SO_4, extract in column with hexane; Florisil column, elute with 5% water–CH_3CN; partition with hexane–dichloromethane; Florisil column, elute with hexane–ether, defat with H_2SO_4 on silica gel; alumina column, elute with 20% CH_2Cl_2–hexane	^{13}C-TCDD 5.0 ng ≡ 200 ppt	values given are mean of 2 GC-MC injections; ratios of 320/322 and 324/332 close to standard
12	10 g fish, digest with conc. HCl, extract with hexane; defat with H_2SO_4 on silica; basic alumina, elute with CH_2Cl_2; $AgNO_3$ on silica gel; basic alumina; HPLC reverse phase-elute with methanol	^{13}C-TCDD 1.0 ng ≡ 100 ppt	2,3,7,8-criteria: (a) retention time, (b) isotopic ratios, (c) signal/noise more than 3; separate run for native and fortified TCDD; possible other TCDD peak reported for sample A

to 15 g) of fish extracts were injected onto the GC columns. As a result the cleanup efficiency had to be greater when large amounts of the final extract were injected. All laboratories used m/z values of 320, 322 for native TCDD plus either 328 (^{37}Cl) or 332-334 (^{13}C) for the internal standard. In many cases additional information (higher resolution, several ions, different GC

Table 3. Summary of MS determination techniques for 2,3,7,8-TCDD in fish

Lab. No.	GC	MS (all EI)	Detection limit, std (pg); detection limit, method (pg/g); amt fish extract injected (g)	Method specificity
1	fused silica OV-101 35 m	Finnigan 4023 MID-12 ions 257, 259, 261, 305, 307, 310, 321, 322, 324, 326, 332, 334	2, 8, 15	isomer specific for 2,3,7,8-TCDD
3	fused silica SE-54 15 m	Varian Mat 311A res. 1000 320, 322, 332	0.1, 2–5, 0.5–1.0	2,3,7,8 plus max. 3 other TCDD isomers confirmed on VG-ZAB instrument at res. 10 000 for m/z 257, 320, 322 on 30 m SE-54 column
4	WCOT SE-30 30 m	Varian Mat 311A res. 8000 320, 322, 328	1.0, 3–9, 0.5	several GC columns run (OV-17, SE-52, SE-30)
5	fused silica DB-5 30 m	Finnigan 4000 320, 322, 324, 326	40, 3–5, 15	2,3,7,8 possibly 1237/1238
6	WCOT SE-30 30 m	Finnigan 4023 320, 322, 324	10–15, 5, 2	not isomer specific
7	WCOT OV-275 50 m	Kratos MS-50 30C ppm scan at res. 10 000 for 320, 322, 328	6–12, 3–5, 5–6	isomer specific for 2,3,7,8-TCDD
9	fused silica SE-54 30 m	Hewlett-Packard 5992 320, 322, 334	11, 5, 1.5–4.5	several GC columns used for confirmation
12	fused silica DP-1 30 m	Hewlett-Packard 5992 320, 322, 324, 334	3–4, 10, 2.0	2,3,7,8 plus up to 2–3 other TCDD isomers

columns) was reported. Detection limits in the fish samples were similar, ranging between 1 and 10 ppt. Two of the methods were shown to be isomer specific for 2,3,7,8-TCDD with respect to all 22 TCDD isomers and most procedures claimed specificity of 2,3,7,8-TCDD plus up to 2–3 other isomers.

TCDD Standard

Three of the reporting laboratories compared their in-house standards with that supplied by the FDA. One was 10% higher, one 8% lower, and the third within 3% of the FDA standard. From this somewhat limited data, there did not appear to be a gross discrepancy between the supplied standard and others in current use.

Table 4. Effect of freeze-drying on TCDD levels in fish

Sample	Isotope and fortification level	Nonfreeze-dried	Freeze-dried
	Radioisotope Experiment[a]		
Smelt	^{14}C, 43 ppt	93	90
	^{3}H, 15 ppt	84	85
	Entire Method[b]		
A		58	64
C		37	31
D		33	29

[a] Recovery (%) of duplicate determinations.
[b] ppt found.

Concentration of 2,3,7,8-TCDD in Fish Samples

The concentration of TCDD reported by all 8 laboratories is summarized in Table 5. Two of the values reported by laboratory 6 were outliers and, because they had apparent difficulties with their method both in this and a previous study of TCDD in fish, their results were not used to calculate the average and variation. Results of sample A from laboratory 1 and sample B from laboratory 12 were also judged to be outliers using Dixon's test (total outliers 18% of reported results). These values are not included in the average values reported of 61.2, 30.4 and 32.3 pg/g for samples A, C, and D, respectively. Sample B was a blank showing no detectable levels of TCDD. The relative standard deviations of samples A, C, and D are 13.9, 18.4, and 25.4%, respectively, and are surprisingly low for pg/g levels of a contaminant in a biological

Table 5. Concentration of 2,3,7,8-TCDD in fish samples from interlaboratory study (single determinations, pg/g (ppt))

Lab No.	Sample A	B	C	D
1[a]	104[b]	ND[c] (10)	35	45
3	58	ND (1.3)	37	33
4[d]	49	ND (2)	23	19
5[e]	58	ND (1)	34	38
6	ND (5)[f]	ND (5)	51[f]	55
7	72	ND (2.3)	25	32
9	70	ND (5)	33	27
12	60	37[g]	26	32
Av.[h]	61.2	3.6	30.4	32.3
SD	8.5	—	5.6	8.2
CV, %	14.0	—	18.4	25.3
n	6	6	7	7

[a] Also reported GC–EC values of 103, ND (10), 39, 37 pg/g, respectively for samples A, B, C, and D.

[b] Value given judged to be an outlier by Dixon's test (ref. 10); recovery of this sample was judged by the analyst to be high (74%) so an average recovery (51%) was used to calculate value given.

[c] Not detected followed by detection limits, pg/g, in parentheses.

[d] Also reported higher values of 58, ND (2), 37, 38 pg/g, respectively, for acid-base method; these values are closer to average than neutral method preferred by the analyst.

[e] Confirmed by atmospheric pressure–negative chemical ionization GC–MS on same extract with values of 54, ND (2.3), 32 and 31 ppt, respectively.

[f] Value given judged to be outlier (ref. 10).

[g] Value given judged to be an outlier (ref. 10); subsequent analysis showed a value of ND (10) pg/g.

[h] Does not include any outliers or values from laboratory 6.

Table 6. Percent recoveries of internal standard TCDD in the interlaboratory round robin study

Lab No.	Av.[a]	SD	RSD, %
1[b]	57.0	11.6	20.4
3[c]	69.0	16.6	24.1
4[d]	80.0	12.3	15.4
5[c]	82.8	19.0	23.0
7[d]	35.3	5.2	14.7
9[c]	74.6	18.4	24.7
12[c]	81.8	14.5	17.7
Av.	68.6	17.3	25.2
Range	29–109		

[a] Each value represents the average of 4 reported values.

[b] Fortified duplicate with native 2,3,7,8-TCDD.

[c] ^{13}C-2,3,7,8-TCDD.

[d] ^{37}Cl-2,3,7,8-TCDD.

sample. Detection limits reported by each of the laboratories according to its own criteria are also shown in Table 5.

A compilation of collaborative studies (11) carried out by AOAC over many years has shown that the coefficient of variation of analytical methods varies inversely with the concentration being measured. At ppm levels, the total variation is between 10 and 20%; in the ppb region it can be more than 40%. The results reported here, although not a collaborative study, are for analytical values in the low ppt region and show a total variation of 14 to 25%. Thus the trend of higher variation at lower concentration reported by Horwitz (11) does not appear to apply in this case. Part of the reason for the lower than expected variation may be due to the use of an isotope as internal standard to correct for losses. In addition, those laboratories that did report 2,3,7,8-TCDD values all had experience in this type of analysis. Five of the 13 participating laboratories did not report for reasons discussed below and of course their values could not be included.

One laboratory reported GC–EC results on the same sample extract which were quite similar to their GC–MS results, indicating the possibility of using this alternative mode of detection for screening purposes before confirmation of positive extracts by GC–MS. Another laboratory reported values using 2 different methods (a neutral extraction and an acid–base method) but indicated preference for the neutral method because it had fewer potential interfering peaks on the GC–MS. The values from the neutral method differed more from the overall average TCDD values than did those of the acid-base method. Most of the data produced by this laboratory on fish has been with the latter method. In one case a laboratory confirmed the concentration of TCDD in the final extract by using the newer technique of atmospheric pressure negative chemical ionization (AP-NCI)-GC–MS with reported values very close to the GC–MS results obtained by electron impact ionization.

Recoveries

Recoveries of the internal standard (either carbon-13 or chlorine-37) of 2,3,7,8-TCDD are given in Table 6 for 6 of the laboratories which used this method for quantitation as well as the laboratory which fortified duplicate samples with native 2,3,7,8-TCDD. The range of most of the average recovery values is 57 to 82% with a relative standard deviation of about 25%. The range of individual values (29–109) is wide and points to the need and usefulness of an internal standard for quantitation of low levels. One laboratory had low but relatively consistent re-

Table 7. Analysis of variance of results (ppt) on 2,3,7,8-TCDD in fish samples C and D, according to Youden (10)

Statistic	Sample C	Sample D
Av.	30.4	32.3
SD	5.59	8.16
$S_d^2 = 2\,S_b^2 + S_r^2$		
	Variance	SD
Total S_d^2	77.45	8.80
Systematic S_b^2 between lab.	28.53	5.34
Precision S_r^2 random	20.40	4.52

F-test = $S_d^2/S_r^2 = 3.80$; df = 6, 6; $F = 4.28$ for $P = 0.05$.

coveries for its method and their corrected results by using an internal standard still were close to the average of the other laboratories. Cairns et al. (12) have expressed a similar sentiment on the use of internal standards.

Statistical Analysis

Fish samples C and D contained similar concentrations of 2,3,7,8-TCDD and were used to calculate a measure of the variation between laboratories (systematic) and within laboratories (random) according to the method of Youden (10) using differences and totals. As shown in Table 7, the variance between laboratories (reproducibility) was greater than within laboratories (repeatability), with an F-test that was, however, not significant at the 95% level for this sample size.

Again, using samples C and D, a comparison was also made between 3 separate factors to see if there were any significant differences using the Student's t-test between selected treatments. Although the data are somewhat limited, there was no difference ($P < 0.05$) in concentration of TCDD in samples C and D from those methods which used (a) digestion (2) or extraction (5), (b) high (3) or low (4) resolution mass spectrometry, and (c) specific (4) or non-specific (3) separation. These samples are not, strictly speaking, a matched pair; therefore differences for example in the amount of interferences could possibly affect these conclusions.

Problems

Three laboratories (2 reporting, one non-reporting) expressed difficulties and reservations in the analysis of freeze-dried oily fish samples. Two of the problems stemmed from emulsions and a third from an assumed tissue polymerization and column blockage. Our desire to use this method of sample preparation derives from the ease of handling, storing, and shipping, particularly when involving many laboratories and more than one country (customs inspection). One non-reporting laboratory had problems with persistent interference peaks particularly from sample A. Three laboratories began the analysis but did not finish because of problems with funding.

Several collaborators reported technical difficulty with their mass spectrometers with resultant time delays. This problem underlined the difficulty of dioxin determination, which depends solely on an expensive highly technical procedure for measurement. One laboratory had a problem with the TCDD standard from a safety viewpoint because the level provided was above that permitted in their laboratory.

All laboratories commented on the refractory nature of fish samples A, C, and D with their high lipid content and high levels of other chemical contaminants. Chloronaphthalenes, PCB, and chlorodibenzofurans were also detected in these fish. In some cases, these compounds can interfere with TCDD determination. No other TCDD isomer was consistently found in the fish samples except the 2,3,7,8 isomer.

Precision

Sample C has been analyzed by the HPB laboratories on 5 separate occasions over a period of 8 months. The values found ranged from 22 to 44 ppt with an average of 34.2 ± 8.2, with a relative standard deviation of 24.1%.

Conclusion

Most of the reporting laboratories had the capability to analyze low levels (<100 pg/g) of 2,3,7,8-TCDD in fish in the 3 positive samples. The relative standard deviation of the analytical values varied between 14 and 26% for 3 of the samples. Considering that each laboratory used a different methodology and MS instrument and that the level determined was in the pg/g range, this variation is surprisingly low. More variation in the reported values was found between laboratories than within but the difference was not significant. More limited data indicated no differences in reported values for 2,3,7,8-TCDD for those methods which used either complete digestion or solvent extraction, high or low resolution mass spectrometry, and specific or non-specific methods. Recoveries of internal standard were quite variable (although most were over 70%) and indicated the necessity for this technique to obtain precise results.

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Capillary Gas Chromatographic-Mass Spectrometric Determination of Individual Chlorobiphenyls in Technical Aroclors

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Six technical Aroclors were separated into individual chlorobiphenyls on fused silica capillary columns. Fifty-three chlorobiphenyl standards were used to ascertain structures of a number of compounds in the technical mixtures by mass spectrometry. Results are compared with reports in the literature. Percentages of identified compounds are given, and the presence of unidentified chlorobiphenyls is indicated by the number of chlorine atoms, in order of compound elution from the column.

Since their discovery in the middle of the 1960s (1), it has been proven that polychlorinated biphenyls (PCBs) are present in organisms all over the world. Many publications have been devoted to identification and measurement of PCBs. In 1974, Jensen and Sundström (2) stated that the determination of individual chlorobiphenyls in a complex mixture is a prerequisite for the proper understanding of the conversion that these substances undergo in the environment. Because we are interested in the behavior of PCB compounds in the food chain, methods based on comparison of peaks with corresponding retention times in samples and technical Aroclors (3-6) or based on conversion into decachlorobiphenyl (7, 8) are not useful.

Although the use of capillary columns greatly improved separations of chlorobiphenyls (9-11), the fact that only a small number of the possible 209 individual chlorobiphenyls are commercially available is a disadvantage. From a practical point of view, we are only interested in the presence of these commercially available chlorobiphenyls in technical mixtures and real-life samples. This paper describes only the analysis of technical Aroclors.

Experimental

Apparatus

Analysis was performed with a Finnigan 4000 quadrupole mass spectrometer and INCOS data system. Mass spectra were linearly scanned every second from mass 100 to mass 500. Emission current was at 200 mA and electron energy at 70 eV.

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Finnigan 9610 gas chromatograph was equipped with a 25 m × 0.22 mm fused silica capillary column coated with CP Sil 5 (SE 30), film thickness 0.1 μm (Chromapack, PO Box 3, Middelburg, The Netherlands). Conditions were linear helium gas velocity ca 30 cm/s; injection port temperature 220°C; column oven programmed from 80 to 240°C: initial hold time 4 min, program rate 10°/min to 150°C and from 150 to 240°C at 3°/min, final hold time ca 15 min.

Reagents

(a) *Technical mixtures.*—Aroclors 1221, 1232, 1242, 1248, 1254 (Lot KL-417), and A1260 (Lot KK-1003).

(b) *Chlorobiphenyl standards.*—See Table 1.

(c) *Internal standard.*—Pentachloroaniline (PCA).

Analysis of Standard Solutions

Technical Aroclors 1221, 1232, 1242, 1248, 1254, and A1260 were separately dissolved, together with PCA, and diluted with isooctane so that 1 μL contained 100 ng Aroclor and 1 ng PCA.

Available chlorobiphenyl standards were also dissolved in isooctane together with PCA to obtain a concentration of 1 ng/μL (compounds 1, 3, and 4, 10 ng/μL; No. 15, 5 ng/μL).

Gas chromatographic separation started with a splitless 5 μL injection of standard solutions in a small plug in the capillary. The solvent was allowed to elute. After 3 min, the splitter was opened, and 4 min after injection, the temperature programmer was started under the conditions previously described.

Results

Table 1 shows a systematic numbering of the chlorobiphenyl compounds, according to Ballschmiter and Zell (12). In the figures, tables, and text, these numbers are used to designate the corresponding chlorobiphenyls.

Figures 1, 2, and 3 show total ion current for Aroclors 1221, 1254, and A1260. The Aroclor mixtures and the chlorobiphenyl standards were

Table 1. Chlorobiphenyl standards in isooctane

Peak[a]	Structure	Peak	Structure	Peak	Structure
1	2-	84	2,3,6-2′3′	151	2,3,5,6-2′5′
3	4-	87	2,3,4-2′5′	153	2,4,5-2′4′5′
4	2-2′	95[b]	2,3,6-2′5′	154	2,4,5-2′4′6′
5	2,3-	97	2,4,5-2′3′	155	2,4,6-2′4′6′
7	2,4-	101	2,4,5-2′5′	156[b]	2,3,4,5-3′4′
8	2-4′	103	2,4,6-2′5′	170	2,3,4,5-2′3′4′
10	2,6-	105	2,3,4-3′4′	180[b]	2,3,4,5-2′4′5′
15	4-4′	118	2,4,5-3′4′	185	2,3,4,5,6-2′5′
18	2,5-2′	119	2,4,6-3′4′	187[b]	2,3,5,6-2′4′5′
28	2,4-4′	121	2,4,6-3′5′	194	2,3,4,5-2′3′4′5′
31	2,5-4′	128	2,3,4-2′3′4′	201[b]	2,3,5,6-2′3′4′5′
33	3,4-2′	129	2,3,4,5-2′3′	202	2,3,5,6-2′3′5′6′
44	2,3-2′5′	132[b]	2,3,4-2′3′6′	206	2,3,4,5,6-2′3′4′5′
47	2,4-2′4′	136	2,3,6-2′3′6′	209	2,3,4,5,6-2′3′4′5′6′
49	2,4-2′5′	137	2,3,4,5-2′4′		
52	2,5-2′5′	138	2,3,4-2′4′5′		
66[b]	2,4-3′4′	140	2,3,4-2′4′6′		
70	2,5-3′4′	141	2,3,4,5-2′5′		
72	2,5-3′5′	149[b]	2,3,6-2′4′5′		
77	3,4-3′4′				

[a] Peak numbering according to Ref. 12.
[b] Gift from C. A. Wachtmeister, Stockholm University, Sweden.

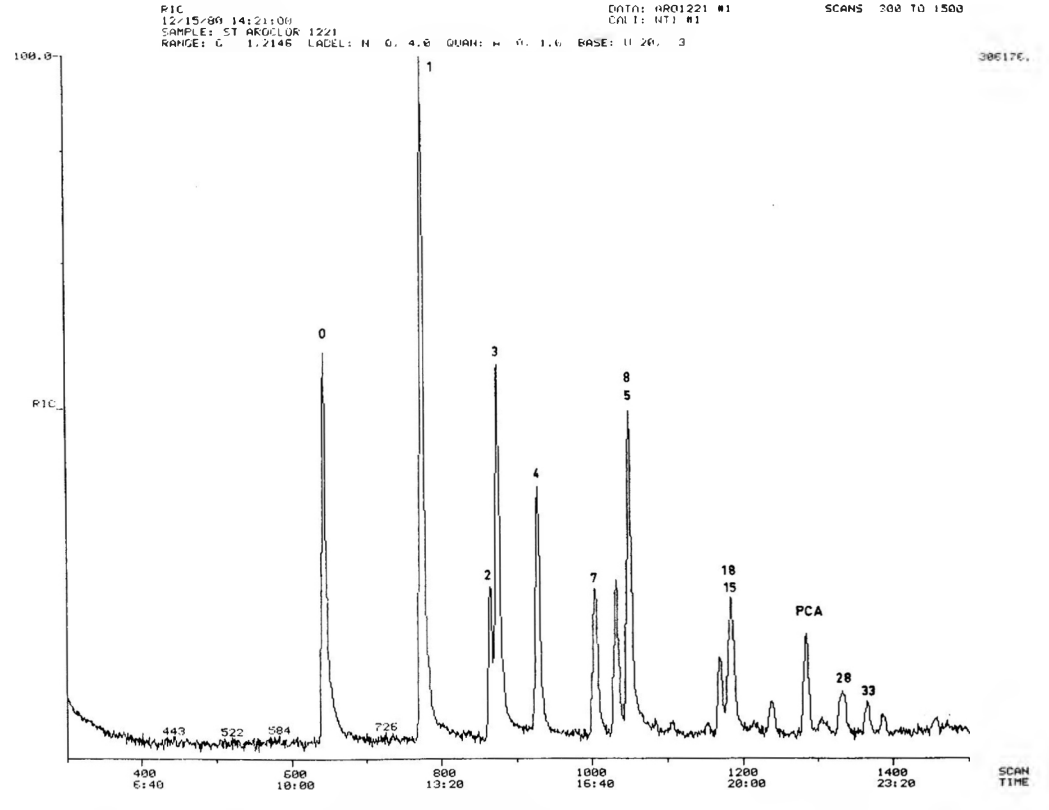

Figure 1. Total ion current of Aroclor 1221. Major peaks numbered according to Ref. 12.

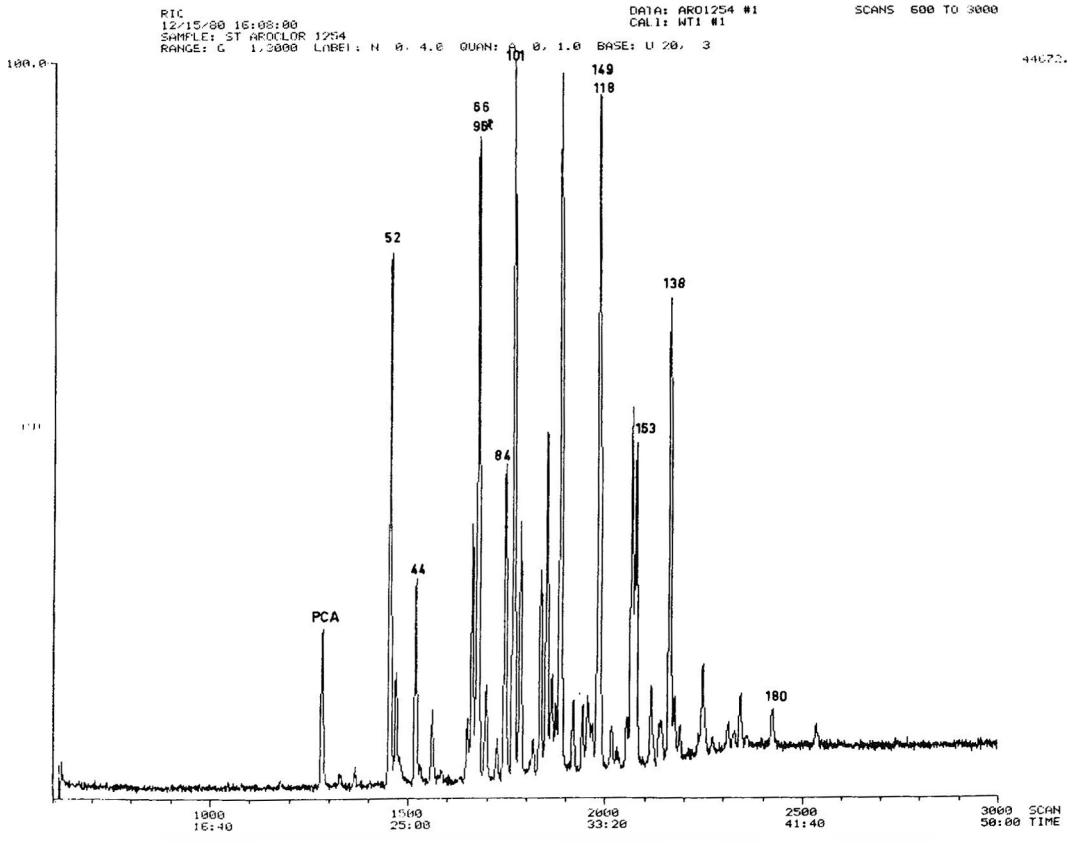

Figure 2. Total ion current of Aroclor 1254. Major peaks numbered according to Ref. 12.

also analyzed on a comparable capillary column connected with an electron capture detector. In that way, it was possible to measure the contribution of each available chlorobiphenyl to the total Aroclor mixture. It must be noted that the composition of the Aroclors can differ from batch to batch. Given content for each chlorobiphenyl is calculated for our Aroclor mixtures.

Six technical Aroclors and available chlorobiphenyl standards were analyzed by gas chromatography–mass spectrometry (GC–MS) at the end of 1980. Since that time more individual chlorobiphenyls have become available to us and their presence in the technical mixtures was also checked.

A total of 53 individual chlorobiphenyls (Table 1) were analyzed by GC-MS.

Table 2 shows results for the 6 technical Aroclors. In order of increasing relative retention time (PCA = 1.000), the number of chlorine

Table 2. GC-MS determination of individual chlorobiphenyls (% w/w) in 6 technical Aroclors[a]

RRT (PCA = 1.000)	No. chlorine atoms found	Code No.	Aroclor 1221	1232	1242	1248	1254	1260	Remarks
0.501	0	0	+	+	+	+	—	—	
0.589	1	1	22.8	15.8	+	+	—	—	
0.663	1	2	+	+	—	+	—	—	1
0.671	1	3	10.8	6.7	+	+	—	—	
0.715	2	4	7.2	7.0	3.1	4.6	—	—	2
0.777	2	7	1.8	1.6	0.2	0.7	—	—	
0.799	2	—	+	+	+	+	—	—	
0.814	2	5/8	(7.8)	(4.0)	(3.6)	(4.5)	—	—	3

Table 2. (cont'd)

RRT (PCA = 1.000)	No. chlorine atoms found	Code No.	Aroclor 1221	1232	1242	1248	1254	1260	Remarks
0.858	3	—	—	—	+	+	—	—	
0.911	2	—	+	+	+	+	—	—	
0.922	2,3	15/18	(1.2)	(4.4)	(10.5)	(9.3)	—	—	4
0.946	3	—	—	+	+	+	—	—	
0.964	3	—	+	+	+	+	—	—	
1.016	3	—	—	+	+	+	—	—	
1.023	3	—	—	+	+	+	—	—	
1.039	3	28/31	(0.8)	(4.3)	(9.6)	(9.0)	—	—	5
1.065	3	33	+	+	+	+	—	—	
1.081	3	—	+	+	+	+	—	—	
1.098	4	—	—	+	+	+	—	—	
1.117	4	—	—	+	+	+	—	—	
1.136	4	52	0.2	1.3	2.1	2.7	3.5	0.5	
1.149	4	49	—	1.1	2.1	2.5	0.8	—	
1.160	4	—	—	+	+	+	—	—	6
1.188	4	44	—	1.4	2.6	3.1	1.5	—	
1.197	4	—	—	+	+	+	—	—	
1.222	4	72	—	2.9	3.3	3.0	0.5	—	
1.241	4	—	—	+	+	+	+	—	
1.266	4	—	—	—	+	+	+	—	
1.277	4	—	—	—	+	+	—	—	
1.290	4	—	—	+	+	+	+	—	
1.300	4	70	—	1.5	2.7	3.4	2.3	—	
1.308	4;5	66;95	—	(1.9)	(3.3)	(4.3)	(7.3)	(3.4)	7
1.329	4;5	—	—	+	+	+	+	—	8
1.347	4	—	—	+	+	+	+	—	
1.360	5	84	—	+	+	+	+	+	9
1.377	5	101	—	0.5	0.4	1.0	7.7	3.8	
1.393	5	—	—	+	+	+	+	+	
1.417	5	119	—	—	—	+	+	—	
1.436	5	97	—	—	0.1	0.4	2.2	0.4	
1.445	5	87	—	0.2	0.2	0.5	3.6	0.8	
1.454	5	—	—	+	+	+	+	—	
1.460	4	—	—	+	+	+	—	—	
1.465	6	136	—	—	—	—	1.1	1.8	10
1.470	5	—	—	+	+	+	+	+	
1.498	5	—	—	+	+	+	+	—	
1.514	6	151	—	—	—	—	1.1	3.8	
1.520	6	—	—	—	—	—	+	+	
1.533	6	—	—	—	—	—	+	+	
1.545	5;6	118;149	—	+	+	(0.1)	(5.4)	(10.7)	11
1.572	6	—	—	—	—	—	+	+	
1.601	6	—	—	—	—	—	+	+	
1.614	6	132	—	+	+	0.4	5.0	+	
1.619	6	153	—	+	+	0.2	6.0	9.8	
1.649	6	141	—	—	—	+	1.7	3.6	
1.666	6	—;137	—	—	—	—	(0.5)	(0.6)	12
1.685	6	138	—	—	—	0.2	7.4	8.8	
1.695	6	—	—	—	—	—	+	+	
1.706	6	129	—	—	—	—	0.2	1.2	
1.745	7	187	—	—	—	—	0.7	6.3	
1.751	6	128	—	—	—	—	1.6	0.6	
1.757	7		—	—	—	—		+	
1.785	7		—	—	—	—		+	
1.800	6		—	—	—	—		+	
1.812	7		—	—	—	—		+	
1.826	7;6	—;156	—	—	—	—	+	+	13
1.835	8	—	—	—	—	—	—	+	
1.890	7	180	—	—	—	—	1.4	11.8	
1.975	7	170	—	—	—	—	0.8	4.2	
2.029	8	201	—	—	—	—	—	4.0	
2.162	8	194	—	—	—	—	—	2.8	
2.271	8	206	—	—	—	—	—	0.7	

[a] Columns 4–9: + = present, but not quantitated; — = not present. See *Results* for further explanation and discussion of data identified in last column, "Remarks."

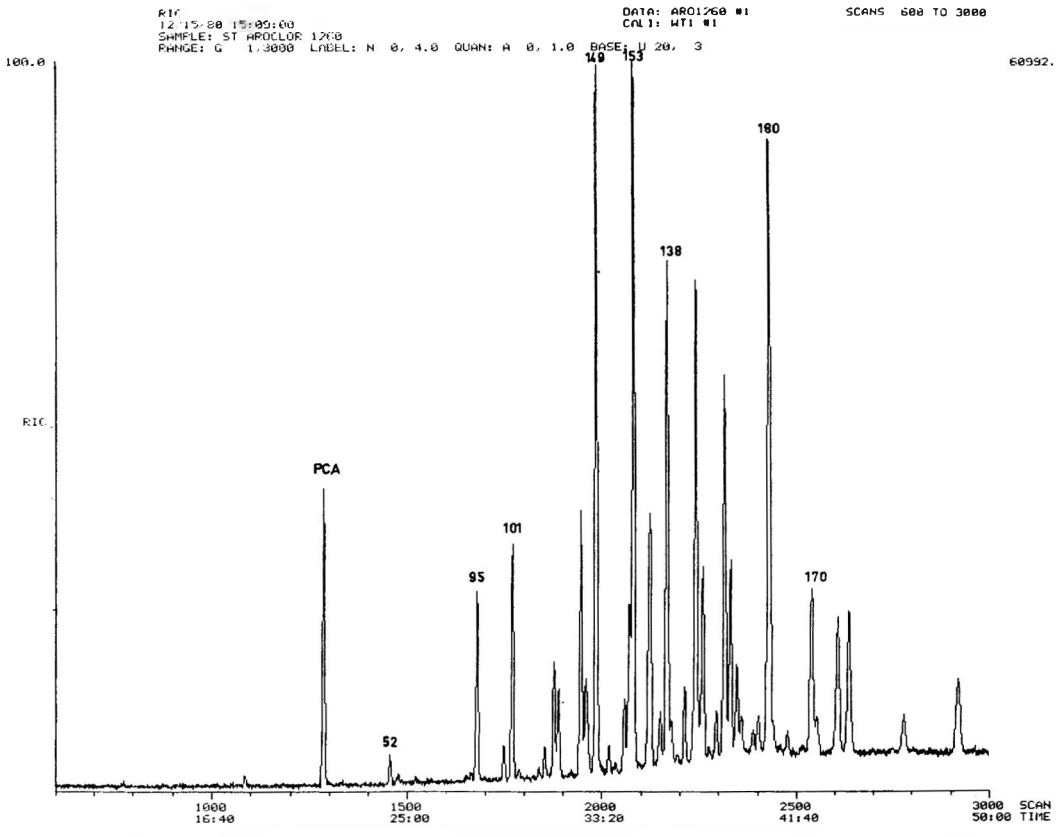

Figure 3. Total ion current of Aroclor 1260. Major peaks numbered according to Ref. 12.

atoms measured by MS is given for every chlorobiphenyl detected in the technical mixture. (For information, the relative retention times for HCB and *p,p'*-DDE are 0.843 and 1.456, respectively.)

When there was also agreement with the retention time of a specified individual chlorobiphenyl standard, this is indicated in the third column by means of the code number and in columns 4–9 with + or the content (% w/w) in the Aroclor mixtures. The symbol + in columns 4–9 means that a certain chlorobiphenyl is present with the number of chlorine atoms indicated in column 2, but no quantitation was carried out, often because the standard was not available; — indicates that a chlorobiphenyl could not be detected in a technical mixture.

Sometimes more than one chlorobiphenyl standard had the same retention time as the compound in the technical mixtures. This is indicated in the third column of Table 2 by 2 code numbers. An unknown compound that coelutes is indicated by —. A number of compounds could not be identified because the comparable standards were not available; however, the number of chlorine atoms was determined.

The following remarks provide clarification and further information on results in Table 2. See the last column of Table 2 to identify data being discussed.

(1) It is quite obvious that only 3 monochlorobiphenyls exist. From Table 1 it appears that 2 monochlorobiphenyls were available and detected in the technical mixture. The unknown monochlorobiphenyl (RRT = 0.663) can only be the 3-monochlorobiphenyl compound.

(2) Two compounds (4 and 10) have the same RRT, 0.715. One isomer (No. 10) has both chlorine atoms in the same phenyl group in the ortho position and the spectrum shows a very weak $[M-Cl]^+$ fragment (Figure 4). The other compound (No. 4) has in each phenyl group one chlorine atom in ortho position and therefore a strong $[M-Cl]^+$ fragment (Figure 5). According to differences in mass spectra between both

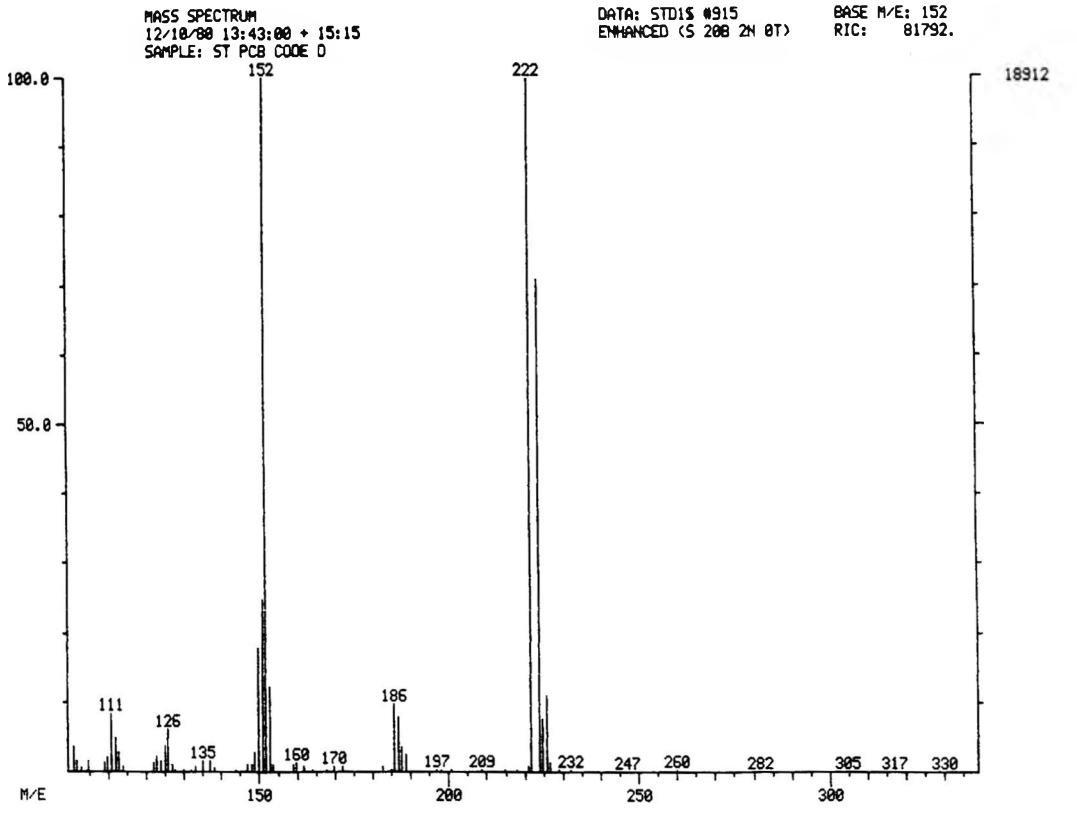

Figure 4. Spectrum of 2,6-dichlorobiphenyl (compound No. 10).

compounds, it appears that in technical Aroclors 1221, 1232 (Figure 6) 1242, and A1248, No. 4 is the major compound.

(3) Two other dichlorobiphenyls (Nos. 5 and 8) also have the same relative retention time. No differentiation can be made from the spectrum, but according to the results of Webb and McCall (13) it is very likely that we have to deal with compound No. 8. The percentages, between parentheses in Table 2, are calculated as if compound No. 8 is present.

(4) Dichlorobiphenyl No. 15 has the same retention time as trichlorobiphenyl No. 18. From the spectra it appears that No. 15 is the main compound present in Aroclor 1221, with a trace of No. 18. In Aroclor 1232, a mixture of both compounds is present; in Aroclors 1242 and A1248, compound 18 is mainly present. Results in Table 2 for all technical mixtures have been calculated as compound No. 18. The MDQ (Minimum Detectable Quantity) (14) on the EC detector for compounds 15 and 18 is 20×10^{-14} and 6×10^{-14} g/s, respectively.

(5) Two compounds, Nos. 28 and 31, elute from the capillary at RRT 1.039. Because the spectra are nearly identical, no distinction can be made in the technical mixture. The reported percentages are calculated as compound 28. (MDQs for the 2 compounds are similar, 3×10^{-14} g/s.)

(6) In the chromatograms the peak width is broadened compared with neighboring peaks. The peak with RRT 1.160 really consists at least of 2 tetrachlorobiphenyls.

(7) The peak with RRT 1.308 consists of a tetrachlorobiphenyl (No. 66) and a pentachlorobiphenyl (No. 95). Compound No. 66 elutes first. In Aroclors 1232, 1242, and A1248, the tetrachlorobiphenyl dominates; in Aroclor 1254, No. 95 dominates. In Aroclor 1260 only No. 95 is present. The percentages in the technical mixtures are calculated as No. 95 (MDQs for the 2 compounds are similar).

(8) The peak with RRT 1.329 is a double peak consisting of a tetra- and a pentachlorobiphenyl. Retention time of compound 121 coincides with

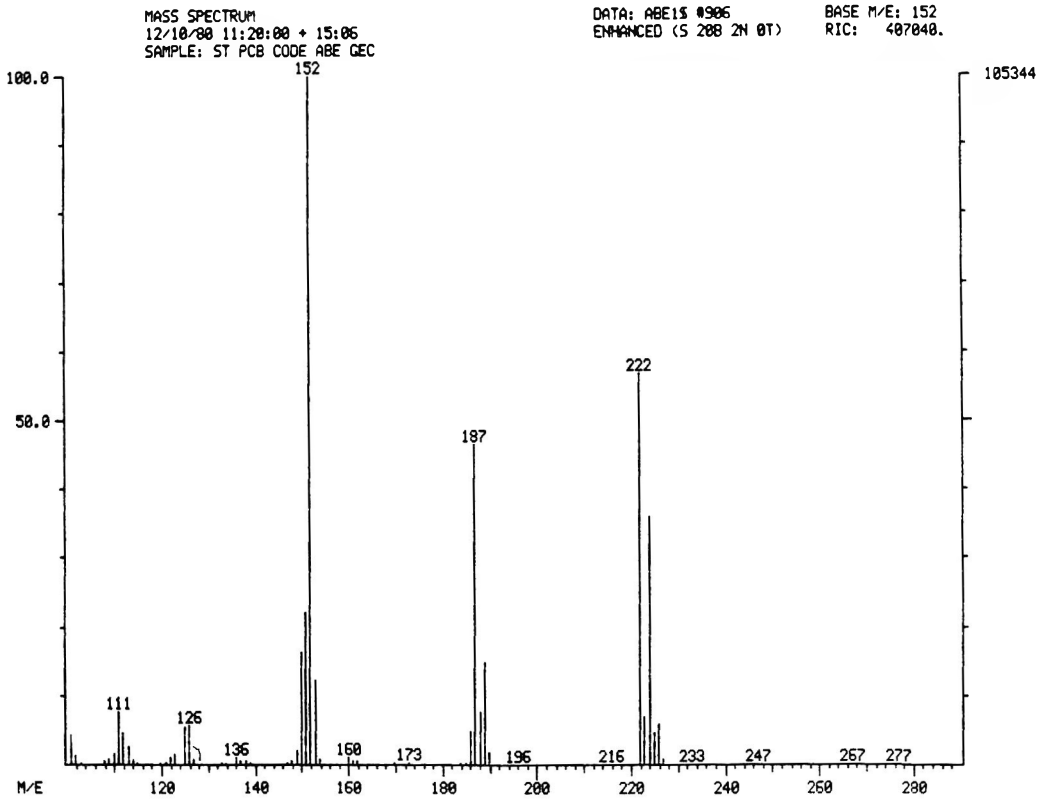

Figure 5. Spectrum of 2-2′-dichlorobiphenyl (compound No. 4).

that for the unknown peak, but from the mass spectra it appears that the unknown pentachloro compound has a strong cluster (m/z 290–298) which is absent in the spectrum of compound 121. Furthermore, it appears that the composition of peak RRT 1.329 differs from one technical Aroclor to the other. The content of the pentachloro compound is nearly equal in Aroclors 1232, 1248, and A1254. In Aroclor 1242, the contribution of this compound is less. There is no such compound in Aroclor 1260.

(9) Compounds 84 and 155 have the same retention times. In all technical mixtures only a pentachloro compound could be detected, e.g., No. 84.

(10) The situation is very complex. The cluster of chromatographic peaks with RRT from 1.460 to 1.470 has a different composition in each technical mixture. In Aroclors 1232, 1242, and A1248, only the tetra- and pentachlorobiphenyls are present. In Aroclor 1254, the penta compound is the major peak compared with the hexa compound (No. 136), and in Aroclor 1260 the hexa compound is the major peak. No tetra compound is present in Aroclors 1254 and 1260.

(11) Again there is a problem of 2 different, but interfering, compounds. In Aroclors 1232, 1242, and 1248, and A1254 the main part is a pentachlorobiphenyl (No. 118) with a trace of a hexachlorobiphenyl. In Aroclor 1260, the hexachloro compound is dominant. According to retention times, compounds 140 and 149 are both possible. The possibility of compound 140 is very small (C. A. Wachtmeister, personal communication). Percentages in the technical mixtures are calculated as No. 149.

(12) Two hexachloro compounds elute with nearly the same retention time. The second is compound 137.

(13) Here there is an interference of a hexa- and a heptachlorobiphenyl. The second eluting compound is hexachlorobiphenyl No. 156 which is the major peak in Aroclor 1254, while in A1260 the heptachloro compound dominates strongly.

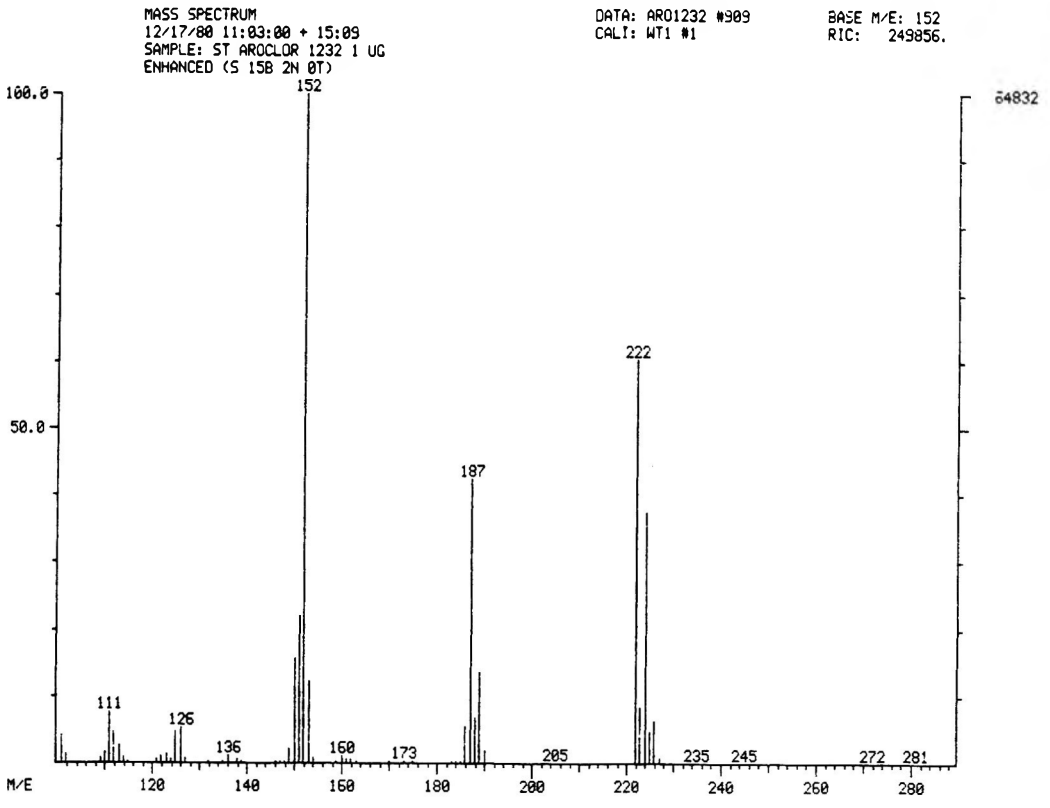

Figure 6. Spectrum of compound with RRT 0.715 in Aroclor 1232.

Discussion

After studying the spectra of each chlorobiphenyl we confirmed the following conclusions (P. A. Leclercq, Laboratory of Instrumental Analysis, Eindhoven University of Technology, The Netherlands, personal communication, 1980): (*1*) Compounds containing more than one chlorine atom with only one chlorine in an ortho position and compounds with no chlorine atoms at all in an ortho position give very weak $[M-Cl]^+$ fragments (Figure 7).

(2) For compounds with 2 chlorine atoms in the ortho position, the intensity of the $[M-Cl]^+$ fragment depends on the distribution of these 2 chlorine atoms over the 2 phenyl rings. When both chlorine atoms are in the ortho position in the same phenyl ring, a weak fragment occurs (Figure 4). When one of the 2 ortho positions in each ring is occupied, a strong $[M-Cl]^+$ fragment results (Figure 5).

(3) Compounds with 2 chlorine atoms in the ortho position show a strong $[M-Cl]^+$ fragment. The effect is stronger than when 2 chlorine atoms are in ortho position, each in one phenyl ring.

(4) Compounds with 4 chlorine atoms in the ortho position behave similar to compounds with only one or no ortho chlorine atom.

Several papers discuss the composition of technical Aroclor mixtures (9, 12, 13). It is often difficult to compare experimental results with the literature because gas chromatographic conditions differ widely.

In a very interesting paper (13), Webb and McCall reported the synthesis of 28 individual chlorobiphenyls and compared their IR spectra with fractions from Aroclor 1254, obtained by preparative gas chromatography. When we compare our results with the results of Webb and McCall, agreement is excellent.

Probably due to better separation, we detect more compounds in the technical mixtures. As far as both institutions had the standards available, compounds identified by Webb and McCall were also confirmed by our investigation.

A recent paper (12) also reported on the composition of Aroclors. Ballschmiter and Zell used many pure chlorobiphenyls and predicted Kovacs indexes for compounds not available to

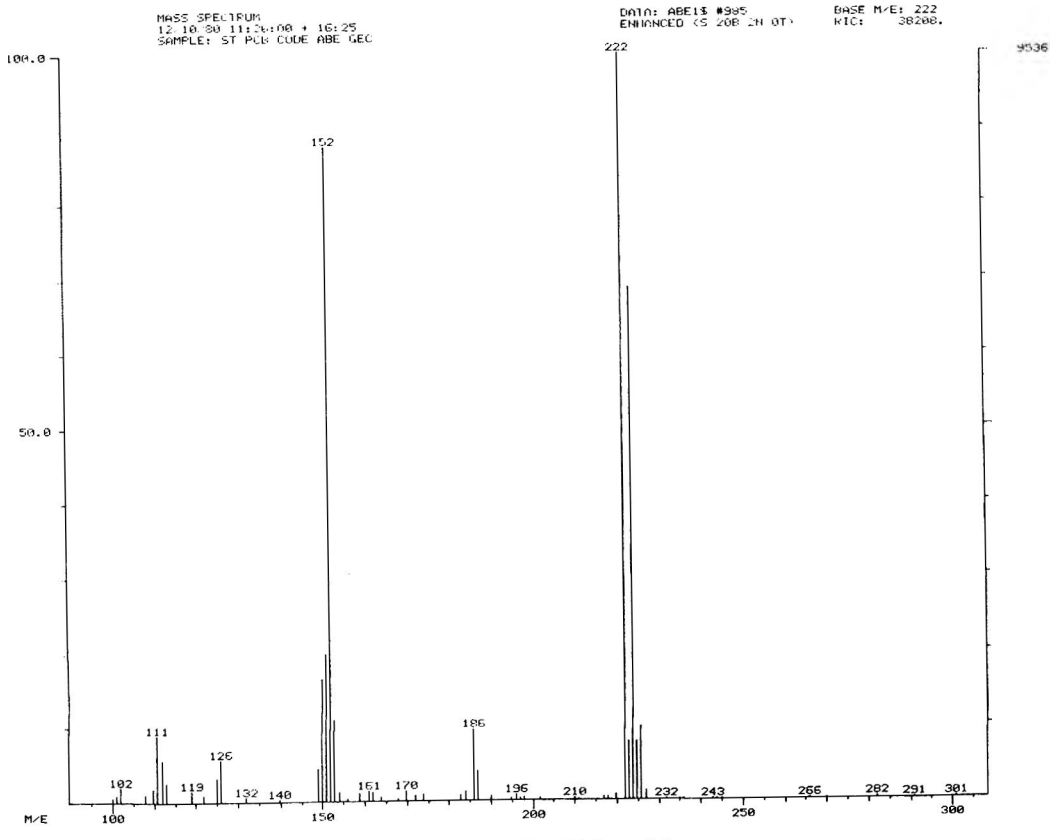

Figure 7. Spectrum of 2,4-dichlorobiphenyl (compound No. 7).

them. Although their gas chromatographic conditions differ from ours, the EC peak pattern shows good agreement.

When we compare their results for Aroclor 1254 with ours, for peaks with an area percentage higher than 1% (for components available to us), we can conclude that identification of compounds available to both of us agree. Some of their predictions are also in full agreement with our results.

Where Ballschmiter and Zell predict or measure only one compound for a given retention time, according to the number of chlorine atoms, in general, our measurements agree. When they measure and/or predict more than one compound there are some agreements with our measurements, but there are also some questions and deviations. (See Table 3 for components shown in order of elution.) For instance at RRT of compound 101, Ballschmiter and Zell predict, in addition to No. 101, a tetrachlorobiphenyl (No. 79); our investigations have proven that there is no evidence for such a compound above the limit of detection, in this case about 0.3%.

At RRT 1.445 (No. 87) they predict the elution of compound 119 and measure No. 87. However, in our study compound 119 elutes before No. 97, which was also correctly measured by Ballschmiter and Zell. The prediction of compound 119 is not justified by our measurements. In the predicted RRT range only hexachlorobiphenyls elute. On the minor compounds (<1%), a few remarks can be made. Agreement exists on compounds 97, 151, and 129; predictions on Nos. 49, 180, and 170 are also correct.

In our system compound 77 would elute at RRT 1.470. In the technical Aroclors, we find an unknown pentachlorobiphenyl. In our study when a tetrachlorobiphenyl should be present (for instance No. 77) it will be about 0.2% at maximum. This agrees with values in the literature (15, 16). Ballschmiter and Zell predict elution of compound 187 after compound 128. We measured No. 128 eluting after No. 187.

Table 3. Comparison of GC–MS results on chlorobiphenyls in Aroclor 1254 (Table 2) with results of Ballschmiter and Zell (12)

This study		Ref. 12
No. chlorine atoms	Code No.	Code No.
—	ND[a]	31[b]
4	52	52
4	49	49[b]
4	44	44
—	ND	37
4	72	64[b];41
4	NI[a]	40
4	NI	ND
4	NI	74[b]
4	70	70
4;5	66;95	96[b];91[b];95
—	ND	102[b]
4;5	NI	89[b];80[b]
4	NI	92[b];84[b]
5	84	90[b]
5	101	79[b];101
5	NI	99
—	ND	150[b]
5	119	83[b]
5	97	97
5	87	119[b];87
5	NI	85[b]
6	136	136
5	NI	77
5	NI	120[b]
6	151	151
6	NI	135[b]
6	NI	NI
5;6	118;149	106;118[b];144[b];149[b]
6	NI	134[b]
6	NI	105;132[b];136
6	132	NI
6	153	153
6	141	141;179[b]
6	NI;137	137;130[b];165[b]
6	138	138
6	NI	NI
6	129	129
7	187	ND
6	128	128;163[b];
—	ND	187[b]
—	ND	159[b]
—	ND	173[b]
—	ND	174[b]
6;7	NI;156	156;177[b]
—	ND	202[b]
7	180	180[b]
7	170	170[b]

[a] ND = not detected; NI = not identified.
[b] Standard not available to authors (12).

Conclusion

When 2 compounds contain the same number of chlorine atoms, it is often difficult to distinguish them by MS alone. Differences in spectra between those compounds are caused mainly by the so-called ortho effect. However, when using a capillary column in combination with MS, a great number of compounds can be irrefutably determined, based on retention time and number of chlorine atoms. For major compounds our identifications agree with the literature. Good predictions on identity can be made when using retention indexes and retention increments as done by Ballschmiter and Zell (12). Many compounds can be measured in technical mixtures by using capillary gas chromatography.

Acknowledgments

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FEEDS

Simultaneous Determination of Protein (Nitrogen), Phosphorus, and Calcium in Animal Feedstuffs by Multichannel Flow-Injection Analysis

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A 3-channel flow-injection procedure was developed, which enables the simultaneous determination of protein, phosphorus, and calcium in a wide range of animal feeds from a single digestion. Samples are digested with a block digestor, diluted, and analyzed at a rate of 82 samples/h. Protein (nitrogen) as ammonia is determined colorimetrically by the indophenol method. Phosphorus and calcium are determined by measuring the absorbances of the molybdenum blue and calcium-cresolphthalein complexes at 660 and 580 nm, respectively. Protein is determined in the range from 0 to 75%, phosphorus in the range from 0 to 6%, and calcium in the range from 0 to 6%. The results obtained do not differ significantly from those obtained by proven manual methods, and considerable time, space, and reagents are saved.

Effective cost control plays an important role in the efficient management of an animal farm. To achieve this goal, recent developments in the formulation of high performance animal feedstuffs have depended largely on the precise definition and control of nutrients. Protein (nitrogen), phosphorus, and calcium are basic components in animal feeds and their levels play an important role in livestock rations for optimum animal performance. Protein is usually calculated by determining the total nitrogen content of the feed by Kjeldahl analyses and multiplying by a factor to convert total nitrogen to crude protein. Phosphorus and calcium, on the other hand, are determined by spectrophotometric methods.

The ever-increasing number of animal feedstuff samples presented for protein, phosphorus, and calcium analyses has led to an increasing demand for rapid and precise chemical procedures. Many analytical methods are available for the determination of protein, phosphorus, and calcium in animal feeds. Many are rather time consuming and tedious when used in routine analysis. Conventional manual methods to obtain the increased throughput in routine laboratories would make heavy labor demands and no doubt would introduce higher total variability due to the large number of operators involved. The simultaneous and automated determination of protein, phosphorus, and calcium in animal feeds would not only save time, but also would contribute to the overall efficiency in this agricultural field.

In 1965, Roach (1) described a segmented automated method for the routine simultaneous determination of calcium and phosphorus in animal feedstuffs. He used a 25% v/v hydrochloric acid solution for digestion and an AutoAnalyzer for processing about 60 samples/h. However, from the operating time given for sample preparation, this was rather slow. Law et al. reported in 1971 (2) on the semiautomation of a Kjeldahl-type method by which total nitrogen and phosphorus can be determined in a wide range of materials from a single digestion of feedstuffs. By using this method they produced a significant increase in the rate of analysis and reliability of results over proven manual methods.

Hambleton and Noel reported in 1975 (3) on the use of a block digestor to completely digest protein in animal feeds. Wall and Gehrke (4) also used a block digestor in conjunction with an automated system for total protein nitrogen. A collaborative study for protein in animal feeds reported by Noel and Hambleton in 1976 (5) used their digestion method (3) and a slightly modified automated method of Wall and Gehrke (4). In 1977, Hambleton (6) developed an automated method for the simultaneous determination of phosphorus, calcium, and crude protein, using the protein digest from a block digestor and without altering the official AOAC method. This automated segmented continuous-flow analysis system gave a sampling rate of 40 samples/h.

Since its introduction in 1974–1975 (7–10) by Stewart et al. and Růžička and Hansen, flow-injection analysis has established itself as an

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analytical technique which is suitable for increasing sample output in most analytical laboratories.

The basic characteristics of flow-injection analysis (FIA) are the absence of air bubbles and the rapid injection of an aqueous sample, either manually through a septum device (11), or automatically by some automatic injection system (10, 12–14) into a continuously moving unsegmented carrier stream of water or reagent solution. The injected sample forms a slug or zone that is processed in a manifold system and transported into a flow-through detection system. Because the sampling period is very short, usually less than 2 s, a high sampling rate can be achieved. This in turn allows the use of a rapidly moving carrier stream which, in contrast to the AutoAnalyzer concept, does not need to be segmented by air. There are no air bubbles and consequently no need to control air bubble size and pattern, and pulsation is reduced because of the absence of air bubbles. The system is highly flexibile in terms of sensitivity; one of the factors, sample volume, for example, can be varied between 1 μL and 25 mL.

The early history of the concept of FIA has been reviewed thoroughly by Stewart (15) and Mottola (16). The basic theoretical background related to this technique has been discussed in detail by Růžička and Hansen (17, 18), Reijn et al. (19), Vanderslice et al. (20), and Painton and Mottola (21). The advantages of this technique have been discussed in comprehensive reviews presented by Růžička and Hansen (17, 18), Betteridge (22), Ranger (23), and van Staden (24). Among the advantages are flexibility, reliability, and reproducibility as well as ease of automation and sample throughput. Despite its great potential for automatic analysis, flow injection has been little applied in the field of animal feeds. Basson and van Staden (25) reported in 1978 an FIA method for the colorimetric determination of calcium in dry-ashed animal feed samples by using cresolphthalein complexone as color reagent and the stable nontoxic 2-amino-2-methylpropan-1-ol reagent as a base solution. An analysis rate of 300 samples/h for calcium in the range 0.5 to 5% with a coefficient of variation of better than 1% was achieved.

The objectives of the present study were to develop automated simultaneous methods for the determination of crude protein, phosphorus, and calcium in a single digested animal feedstuff sample, using the protein digest from a block digestor and the application of multichannel flow-injection analysis.

Chemistry

Protein (ammonia).—The estimation of protein (total nitrogen) is based on the reaction of ammonia with phenol and hypochlorite in an alkaline medium to form a blue complex. The reaction is catalyzed by sodium nitroprusside. The developed color is measured at 620 nm.

The so-called indophenol blue reaction in which ammonia reacts with phenol and hypochlorite to form a blue color in an alkaline medium was first reported by Berthelot in 1859 (26). The reaction was quantitatively used by Thomas in 1912 (27). Since then this method has been successfully used and investigated by several other workers (28, 29) for the determination of ammonia in a number of different materials. The optimum conditions (29) for this reaction have been the subject of numerous investigations and are well established. This method has been automated and used by several workers (6, 29, 30).

Phosphorus.—The determination of phosphorus is based on the complexation of orthophosphate with acidic molybdate. This complex is reduced to molybdenum blue with ascorbic acid. The developed color is measured at 660 nm.

The 2 main colorimetric systems available for total phosphorus determination as orthophosphate are either a molybdenum blue or an ammonium vanadate procedure. The ammonium vanadate procedure is used for relatively high phosphate concentrations whereas the molybdenum blue procedure, because of its higher sensitivity, is normally selected for low phosphate concentrations. However, the system finally chosen had to be capable of analyzing low level phosphate contents (that is, 0–60 mg/L) in the digests, so the more sensitive molybdenum blue method was chosen for experimental work. The reaction conditions for the molybdenum blue reaction are furthermore well established and, with the exception of the particular reducing agent used, present no difficulties. Various reducing agents have been reported for the reduction of the phosphomolybdate complex to molybdenum blue, including hydrazine sulfate (31), sodium sulfite (31), sodium sulfate, hydroquinone, ferrous sulfate, stannous chloride (32), aminonaphthol sulfonic acid (33), and ascorbic acid (34). The use of ascorbic acid in particular resulted in an exceptionally stable baseline with the best color development in the shortest time, making it suitable for flow-injection analysis.

In 1971, Law et al. (2) automated the molybdenum blue phosphate method for the determination of phosphorus in feedstuffs.

Calcium.—Most continuous flow analytical procedures use a procedure similar to that of Kessler and Wolfman (35) for the determination of calcium with cresolphthalein complexone reagent and diethylamine–sodium acetate as a base component measuring the absorbance of a calcium–cresolphthalein complex at 580 nm and pH 12.0. Working at this pH and wavelength gave less interference from magnesium. Gitelman, in 1967 (36), improved this method by introducing quinolin-8-ol to eliminate interference from magnesium and measured the complex at 570 nm. Roach (1) applied this method with a sampling rate of 60 samples/h for the estimation of calcium in animal feeds, measuring the complex at 580 nm and pH 10.7. In 1974, Moorehead and Biggs (37) modified this method by replacing the toxic, volatile diethylamine (pK_a = 11.0) with the more stable, nontoxic 2-amino-2-methylpropan-1-ol (pK_a = 9.6) as a base solution. The cresolphthalein complexone reagent is almost colorless at pH 10 but highly colored at pH 12; therefore, the blank was reduced and the sensitivity was increased. They reported that since the magnesium interference was eliminated by using quinolin-8-ol, it was not necessary to work at the higher pH. In 1978, Basson and van Staden (25) found that the 2-amino-2-methylpropan-1-ol as base gave a sufficiently stable solution for FIA-analysis, which obviates the use of the poisonous potassium cyanide as stabilizer.

METHOD

Principle

Samples are digested in 250 mL calibrated tubes in a block digestor and analyzed automatically by use of the Berthelot (26) reaction for ammonia, the molybdenum blue method (34) for phosphate, and the cresolphthalein complexone procedure (35) for calcium. Absorbances of indophenol blue complex for ammonia, molybdenum blue complex for phosphate, and calcium–cresolphthalein complex are read in 10 mm Hellma-type flowcells at 620, 660, and 580 nm, respectively.

Apparatus

(a) *Block digestor.*—Capable of maintaining 410°C and digesting a series of samples simultaneously in 250 mL calibrated volumetric tubes constricted at top. Block must be equipped with removable shields to enclose exposed areas of tubes completely at or above height of constriction.

(b) *Multichannel flow-injection analyzer.*—(*i*) *Sampling valve systems.*—Three Carle microvolume 2-position sampling valves (Carle Cat. No. 2014), each with 2 identical sample loops of 25 μL volume. A constant stream of samples from animal feed digests was supplied to the 3 sampling valve systems. Carrier streams were supplied by peristaltic pump, and valve systems were synchronized with sampler unit. (*ii*) *Cenco sampler.* (*iii*) *Cenco peristaltic pump.*—Operating at 10 rpm. (*iv*) *Manifold.*—See Figure 1. (*v*) *Spectrophotometers.*—Bausch and Lomb Spectronic 21 DV spectrophotometers (Rochester, NY) equipped with 10 mm Hellma-type flowthrough cells (volume 80 μL), and sensitivity switch selected to high sensitivity (see Figure 1). (*vi*) *Voltage stabilizers.*—Mettler Cl 110 power line filter. (*vii*) *Recorders.*—Of appropriate span (see flow diagram in Figure 1).

Reagents

Prepare all reagents from analytical reagent quality chemicals unless otherwise specified.

Protein

(a) *Alkaline phenol.*—Dissolve 50 g phenol slowly in 100 mL 20% sodium hydroxide solution. Add 100 mL ethanol slowly and mix well. Add 0.5 mL Brij 35 and dilute to 500 mL with water.

(b) *Alkaline hypochlorite.*—Add 10 g sodium tetraborate decahydrate to 50 mL 20% sodium hydroxide solution and 450 mL household bleach solution (containing 3.5% active chlorine). Shake well until dissolved.

(c) *Sodium nitroprusside.*—Dissolve 0.6 g sodium nitroprusside in 500 mL water.

(d) *Nitrogen stock solution.*—Accurately weigh 4.7150 g ammonium sulfate (vacuum-oven dried at 70°C) and dissolve in 500 mL water. Dilute quantitatively to 1 L with water. Solution contains 1000 mg N/L solution.

Phosphorus

(a) *Acidic molybdate.*—Dissolve 3.1 g ammonium heptamolybdate in 500 mL of 0.4 mol/L nitric acid.

(b) *Ascorbic acid.*—Dissolve 5 g ascorbic acid in 200 mL water. Add 1 mL glycerine and 0.5 mL Ultrawet 60 L. Do not add Brij 35 to phosphorus reagents. Dilute to 500 mL with water.

(c) *Phosphate stock solution.*—Accurately weigh 4.3943 g potassium dihydrogen orthophosphate (vacuum-oven dried at 50°C) and dissolve in 600 mL water. Dilute quantitatively to 1 L with water. Solution contains 1000 mg P/L solution (32.29 mg KH_2PO_4/L solution).

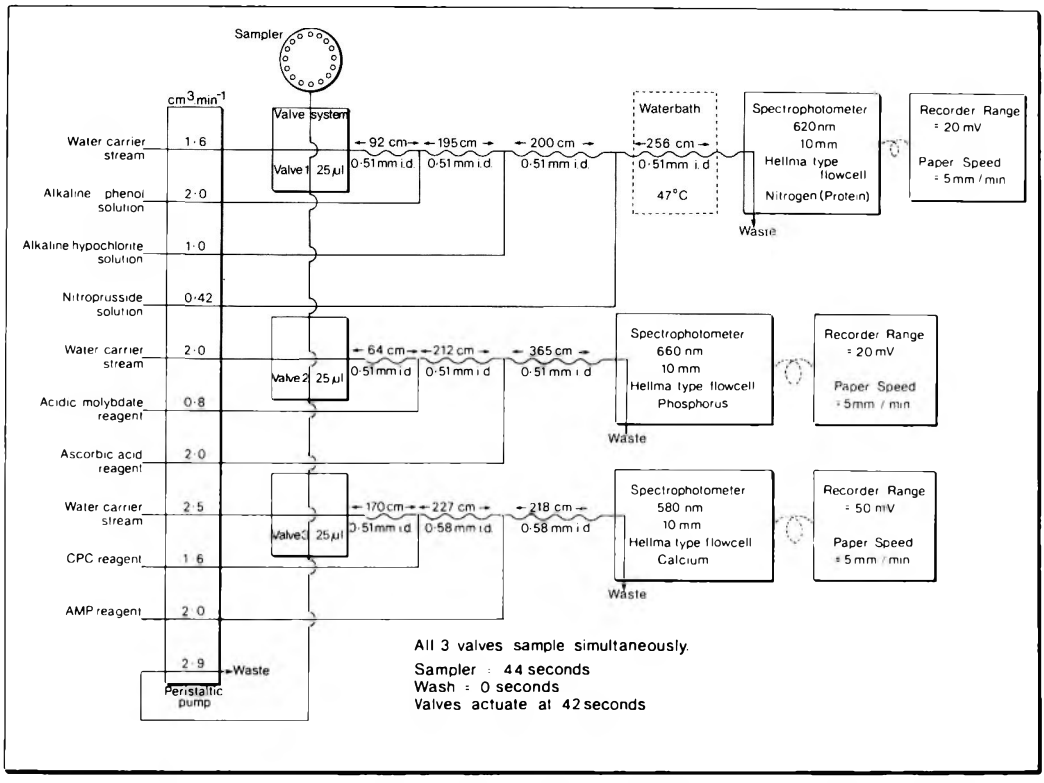

Figure 1. Manifold and flow diagram for simultaneous determination of protein (nitrogen), phosphorus, and calcium on a single digested animal feedstuff sample, using multichannel analysis. Valve loop sizes = 25 µL. Sampling rate 82 samples/h (ca 240 analyses/h). Tube length and id are given in cm and mm, respectively.

Calcium

(a) *Cresolphthalein complexone reagent (CPC).*—Add 0.10 g cresolphthalein complexone and 20 mL concentrated hydrochloric acid to 50 mL water in a 1 L volumetric flask and swirl gently until dissolved. Add 2 g quinolin-8-ol and swirl gently until dissolved. Dilute to 1 L with water. Filter if necessary.

(b) *Base solution (AMP).*—Dissolve 50 g 2-amino-2-methylpropan-1-ol (AMP) in 500 mL water.

(c) *Calcium stock solution.*—Dissolve 1.2490 g Analar grade calcium carbonate carefully in ca 0.1 mol/L hydrochloric acid by adding the acid dropwise until all calcium carbonate has just dissolved. Boil solution a few minutes to remove carbon dioxide. Dilute quantitatively to 1 L with distilled water. The solution contains 500 mg calcium/L solution.

(d) *Standard working solutions.*—Prepare 6 standards by adding volumes of stock standard solutions indicated in Table 1 into 250 mL di-

Table 1. Amounts of standard stock solutions to be diluted to 250 mL

	Stock solution, mL			mg/L		
Tube	Calcium	Phosphorus	Protein (N)	Calcium as Ca	Phosphorus as P	Protein as N
1	5	2.5	5	10	10	20
2	10	5.0	10	20	20	40
3	15	7.5	15	30	30	60
4	20	10.0	20	40	40	80
5	25	12.5	25	50	50	100
6	30	15.0	30	60	60	120

Table 2. Amounts of sample to be weighed according to protein content

Min. and max. protein, %	Sample, g	Min. and max. phosphorus, %	Min. and max. calcium, %
0–12	1.5	0.17–1	0.17–1
3–18	1.0	0.25–1.5	0.25–1.5
3.9–23	0.8	0.31–1.8	0.31–1.8
5–31	0.6	0.42–2.5	0.42–2.5
6–37	0.5	0.5–3.0	0.5–3.0
12.5–75	0.25	1.0–6.0	1.0–6.0

gestion tubes. Add 9 g potassium sulfate, 0.42 g mercury-II-oxide (HgO), and 5 mL concentrated sulfuric acid to each tube. Insert tubes into digestor block preheated to 410°C, place shields around tubes, and digest for 1 h.

After digestion, remove tubes, place in fume hood, and let cool 10–15 min. Dilute contents quantitatively to 250 mL with water and mix thoroughly. Use these solutions for working standards, containing 10, 20, 30, 40, 50, and 60 mg Ca/L solution, 10, 20, 30, 40, 50, and 60 mg P/L solution, and 20, 40, 60, 80, 100, and 120 mg N/L solution. Standards are stable ≧2 months.

Analytical System

Schematic flow diagram for simultaneous determination of protein, phosphorus, and calcium in animal feeds is given in Figure 1. Manifold consists of Tygon tubing with inside diameters as indicated in Figure 1 cut into required lengths and wound around suitable glass tubes with outside diameter of 15 mm. Carrier and reagent streams are supplied at constant flow rates with Cenco peristaltic pump operating at 10 rpm, and sampling valve systems are synchronized with Cenco sampler unit. Single digested animal feed sample taken from turntable of automatic sampler is pumped into sample loops of each of 3 different valve systems by means of peristaltic pump (Figure 1). The 3 samples are injected simultaneously into 3 reaction manifold systems to be chemically processed and detected separately for nitrogen, phosphorus, and calcium, respectively. A 44 s sampling cycle is used between successive samples. The 3-channel system is suitable for simultaneous analysis of nitrogen, phosphorus, and calcium at 82 samples/h (that is ca 240 determinations/h). All 3 valves simultaneously sample 25 μL digested animal feed sample. The 3 valve systems are actuated on a time basis correlated with sampler unit, every 42 s after movement of sampler to next sample.

(a) *Nitrogen manifold.*—The injected 25 μL sample is combined with the water carrier stream pumped at constant rate of 1.6 mL/min. The 2.0 mL/min alkaline phenol solution reagent stream is added and mixed in a 195 cm × 0.51 mm id coil. Alkaline hypochlorite solution reagent is added at rate of 1.0 mL/min and combined in a 200 cm × 0.51 mm id coil. After mixing, sodium nitroprusside solution reagent is added at a rate of 0.42 mL/min and color is developed in a 47°C, 256 cm × 0.51 mm id coil before measurement in spectrophotometer at 620 nm.

(b) *Phosphate manifold.*—The injected 25 μL sample is combined with water carrier stream pumped at constant rate of 2.0 mL/min and mixed in a 64 cm × 0.51 mm id coil. The 0.8 mL/min acidic molybdate reagent solution stream is added and mixed in a 212 cm × 0.51 mm id coil. Ascorbic acid solution is added at a rate of 2.0 mL/min. Mixing and color development takes place in a 365 cm × 0.51 mm id coil before measurement in a spectrophotometer at 660 nm.

(c) *Calcium manifold.*—The injected 25 μL sample is combined with water carrier stream pumped at constant rate of 2.5 mL/min and mixed in a 170 cm × 0.51 mm id coil. The 1.6 mL/min cresolphthalein complexone reagent (CPC) is added and mixed in a 227 cm × 0.58 mm id coil. 2-Amino-2-methylpropan-1-ol (AMP) solution is added at a rate of 2.0 mL/min. Mixing and color development takes place in a 218 cm × 0.58 mm id coil before measurement in a spectrophotometer at 580 nm.

Determination

Weigh finely ground animal feed samples according to amount of protein (see Table 2) into dry digestion tubes. Add 9 g potassium sulfate, 0.42 g mercury-II-oxide (HgO), 5 mL concentrated sulfuric acid, and 2 glass beads to each tube. Insert tubes into digestor block preheated to 410°C, place shields around tubes, and digest 1 h. After digestion, remove tubes and cool 10–15 min in fume hood. Dilute quantitatively to 250 mL with water and mix well. Use these solutions for analysis.

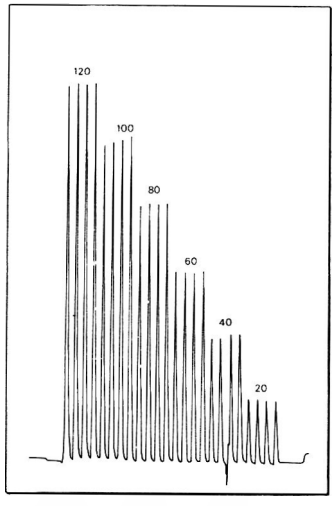

Figure 2. Typical curve for series of standard nitrogen solutions. Numerals = mg N/L.

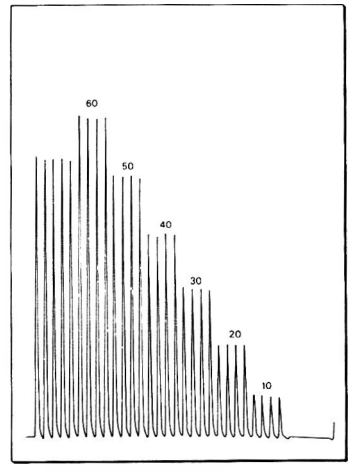

Figure 3. Typical curve for series of standard phosphorus solutions. Numerals = mg P/L.

Place standards in tray in decreasing order of concentration, followed by groups of samples. Proceed and follow each group of samples with series of standards to correct for possible drift, if any. Analyze standards and samples at 82 samples/h.

Prepare standard curve by averaging peak heights of first and second set of standards. Plot average peak heights for standards against nitrogen concentration as N, phosphorus concentration as P, and calcium concentration as Ca in mg/L contained in each 250 mL tube.

$$\text{Protein, \%} = [(\text{mg N/L from graph}) \times 6.25 \times 100]/(\text{mg sample in 250 mL tube} \times 4)$$

$$\text{Phosphorus, \%} = [(\text{mg P/L from graph}) \times 100]/(\text{mg sample in 250 mL tube} \times 4)$$

$$\text{Calcium, \%} = [(\text{mg Ca/L from graph}) \times 100]/(\text{mg sample in 250 mL tube} \times 4)$$

Experimental

Many factors could limit the development of the simultaneous determination of protein, phosphorus, and calcium in a single digested animal feed sample, because any alterations in the official protein method could affect its status. However, these factors have been tested in detailed studies by Law et al. in 1971 (2) and by Hambleton in 1977 (6) and it was shown that these factors did not place any limitations on the segmented automated simultaneous determination of protein, phosphorus, and calcium in a single digested animal feed sample.

Figures 2, 3, and 4 show typical curves for protein, phosphorus, and calcium at a rate of 82 samples/h. The ranges of 20–120 mg N/L, 10–60 mg P/L, and 10–60 mg Ca/L in the digested animal feed sample were chosen because the great majority of animal feed samples fall within these ranges. A recorder paper speed of 5 mm/min and a sampling rate of 82 samples/h were used for the recordings. The samples were introduced in a random order to test carry-over effects. Carry-over from one sample to another is negligible.

The automated portion of the determination of protein (total nitrogen) was based on the determination of ammonia by the well established indophenol blue procedure. The optimum

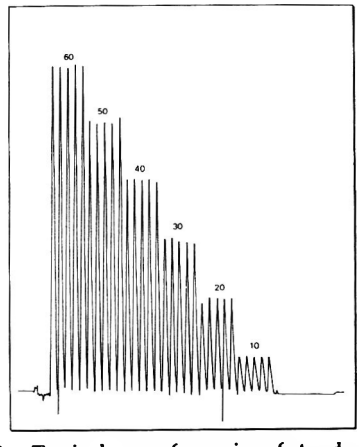

Figure 4. Typical curve for series of standard calcium solutions. Numerals = mg Ca/L.

Table 3. Reproducibility test of the proposed FIA method for protein, phosphorus, and calcium [a]

Sample	Protein, %	RSD	Phosphorus, %	RSD	Calcium, %	RSD
1	4.38	0.69	0.82	0.67	0.55	0.88
2	20.64	0.61	4.67	0.44	1.02	0.76
3	7.49	0.68	2.52	0.57	0.88	0.85
4	15.21	0.59	3.32	0.54	0.60	0.86
5	9.86	0.71	0.98	0.61	2.87	0.66
6	2.82	0.79	0.70	0.69	0.82	0.63
7	4.41	0.82	0.61	0.76	1.60	0.74
8	13.92	0.70	1.08	0.59	5.71	0.51

[a] Mean result of 15 tests in each case.

conditions for this reaction has been the subject of numerous investigations and is well established. Reagents were optimized to give a linear calibration curve in the concentration range of 20–120 mg N/L.

The automated portion of the phosphorus method was based on the complexation of orthophosphate with acidic molybdate and the reduction to molybdenum blue. This was possible because all phosphorus was converted in the digestion procedure to the ortho form. Choice of optimum conditions for the molybdenum blue method has also been the subject of numerous investigations and is well established. Reagents were optimized to give a linear calibration curve in the concentration range of 10–60 mg P/L.

The automated calcium portion of the simultaneous method is an adaption of a method previously used by Basson and van Staden in 1978 (25) which measures the colored calcium–cresolphthalein complex in a highly buffered medium. Cresolphthalein complexone is an alkaline-earth metal complexing dye. Magnesium interference was eliminated by using quinolin-8-ol, and 2-amino-2-methylpropan-1-ol (pK_a = 9.6) was used as the alkalizing agent. The 2-amino-2-methylpropan-1-ol alkalizing agent allows reaction at pH 10.2 where the cresolphthalein complexone is essentially colorless unless calcium is present. This increases the sensitivity. The 2-amino-2-methylpropan-1-ol is nontoxic; the solution is stable enough to obviate the use of poisonous potassium cyanide as stabilizer and does not react with the pump and Tygon tubing.

Experiments to determine the effect of various concentrations of sulfuric acid were conducted. During any digestion, acid concentration may vary from 3 to 6% by volume depending on the amount of sulfuric acid necessary in the digestion of organic matter and the loss through evaporation. Results for phosphorus and ammonia indicated no difference between these limits. However, the complexone calcium reaction was very sensitive to pH change because cresolphthalein complexone is also an acid–base indicator. The use of a concentrated buffer solution overcame the problem.

The interference of mercury on the procedure has been studied before (6) and it was shown that high mercury-II-oxide concentrations did not affect either protein or phosphorus results, but slightly enhanced the color intensity of the calcium–cresolphthalein complex. At low concentrations, as in the digestion procedure, no interference was observed.

Cresolphthalein complexone is used as an indicator for the alkaline earth elements; therefore, magnesium interference must be eliminated in the calcium system by complexing with quinolin-8-ol. This interference has been studied (6) and it was shown that a sample could contain at least 4.5% magnesium before any interference was detected. These results were confirmed.

Experiments were also conducted to determine the effect of calcium precipitation in the proposed method. Studies showed that the added standards in one working solution did not affect the proposed method in the working concentration ranges when compared with standards containing single components.

Results and Discussion

Table 3 shows the results of a reproducibility study of the proposed method on digested animal feed samples for the determination of protein, phosphorus, and calcium (mean result of 15 tests in each case). The relative standard deviations for the 15 tests on an animal feed sample indicate good precision for the proposed multichannel FIA system. The accuracy of the proposed method was also tested by the standard additions method. Known amounts of nitrogen (protein), phosphorus, and calcium were added to animal feed samples which were processed

Table 4. Comparison of results for protein, phosphorus, and calcium contents of animal feeds: proposed method and modified standard addition method

	Protein, %			Phosphorus, %			Calcium, %		
Sample	FIA	Std addn	Rec., %	FIA	Std addn	Rec., %	FIA	Std addn	Rec., %
1	5.89	5.87	99.7	2.06	2.04	99.0	0.54	0.54	100.0
2	21.20	21.16	99.8	3.99	3.99	100.0	1.16	1.13	97.4
3	16.80	16.76	99.8	4.71	4.68	99.4	0.81	0.79	97.5
4	9.34	9.31	99.7	0.61	0.60	98.4	1.89	1.88	99.5
5	5.91	5.89	99.7	1.23	1.21	98.4	2.21	2.17	98.2
6	13.37	13.35	99.9	1.91	1.89	99.0	5.40	5.34	98.9

Table 5. Comparison of results for protein, phosphorus, and calcium contents in animal feedstuffs: proposed FIA method and standard manual methods

	Protein, %		Phosphorus, %		Calcium, %	
Sample	FIA	Kjeldahl	FIA	Vanadate	FIA	AAS
1	1.91	1.87	0.47	0.51	0.68	0.71
2	5.43	5.42	0.63	0.61	3.12	3.14
3	8.67	8.61	0.99	0.98	0.69	0.71
4	11.67	11.60	4.32	4.29	1.09	1.06
5	4.99	4.98	0.64	0.64	0.87	0.86
6	14.31	14.26	3.29	3.31	2.73	2.69
7	5.84	5.79	1.03	1.01	1.99	2.01
8	14.20	14.11	1.92	1.93	5.61	5.65
9	7.93	7.98	0.73	0.69	2.23	2.20
10	7.21	7.23	0.89	0.88	0.56	0.56
11	2.37	2.34	0.58	0.62	0.82	0.87
12	21.85	21.77	4.10	4.14	3.71	3.67
13	3.73	3.74	0.61	0.59	1.31	1.29
14	6.79	6.73	0.93	0.95	0.66	0.69
15	9.26	9.27	1.09	1.11	0.51	0.53

before and after addition (Table 4). It can be seen that the nitrogen (protein), phosphorus, and calcium are fully recovered in each case.

Table 5 shows results obtained for the protein, phosphorus, and calcium contents of a number of animal feedstuff samples with the proposed multichannel FIA procedure and those obtained with standard manual procedures. The proposed FIA method compares favorably with the standard manual methods as seen from Table 4.

The precision of the simultaneous multichannel FIA system was determined by analyzing 15 replicate samples. Relative standard deviations for protein, phosphorus, and calcium were better than 0.8, 0.8, and 0.9, respectively.

Results for a number of routine animal feedstuff samples analyzed by the simultaneous multichannel FIA system and standard manual methods indicate that the former is accurate and precise for the determination of protein, phosphorus, and calcium in animal feeds. No significant differences were found in accuracy and precision.

Conclusion

The 3-channel flow-injection system described here is suitable for the simultaneous photometric determination of protein, phosphorus, and calcium on a single digested animal feedstuff sample at a rate of 82 samples/h (that is approximately 240 analyzed per hour) with a standard deviation of better than 0.8 for protein, 0.8 for phosphorus, and 0.9 for calcium.

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OILS AND FATS

High Performance Liquid Chromatographic Determination of Seven Antioxidants in Oil and Lard: Collaborative Study

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A high performance liquid chromatographic procedure for the determination of propyl gallate (PG), 2,4,5-trihydroxybutyrophenone (THBP), *tert*-butylhydroquinone (TBHQ), nordihydroguaiaretic acid (NDGA), 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) was collaboratively studied by 8 laboratories. The 14 samples analyzed consisted of 10 vegetable oil samples spiked in matched pairs at about 200, 100, and 20 ppm and 4 lard samples spiked in matched pairs at about 100 and 40 ppm for each antioxidant except NDGA which was spiked only at the 2 lower levels in oil. In the method studied, the samples were dissolved in hexane and the antioxidants were partitioned into acetonitrile. The acetonitrile was concentrated and diluted with isopropanol to give isopropanol-acetonitrile (1 + 1). The antioxidants were separated by reverse phase gradient elution and detected at 280 nm. The results from one laboratory were rejected as outlying and were not considered in any calculations. For the remaining 7 laboratories, the overall mean recoveries for PG, THBP, TBHQ, NDGA, BHA, Ionox-100, and BHT were 93.2, 95.1, 95.6, 95.5, 98.3, 95.8, and 84.8%, respectively, and the overall mean coefficients of variation were 5.02, 7.74, 19.3, 4.36, 3.75, 6.33, and 3.45%, respectively.

A high performance liquid chromatographic procedure for the determination of propyl gallate (PG), 2,4,5-trihydroxybutyrophenone (THBP), *tert*-butylhydroquinone (TBHQ), nordihydroguaiaretic acid (NDGA), 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), octyl gallate (OG), and dodecyl gallate (DG) in oils, lards, and shortenings was presented at the 92nd Annual Meeting of the AOAC (1978) and recommended for collaborative study (1). In this procedure the oil, lard, or shortening is dissolved in hexane and the antioxidant is partitioned into acetonitrile. The acetonitrile extract is then concentrated under vacuum, transferred, and diluted with isopropanol to give isopropanol–acetonitrile (1 + 1). An aliquot is injected onto a 3.2 × 250 mm column packed with 10 μm LiChrosorb RP-18; gradient elution from 30% water in acetonitrile to 100% acetonitrile, both solvents with 5% acetic acid, separated 7 of the 9 antioxidants. With the acetonitrile–water system, OG and Ionox-100 co-eluted, however, they could be adequately separated by a water–methanol gradient. This overlap with water–acetonitrile was not considered a serious problem, because Ionox-100 is permitted only in the United States. OG is not permitted in the United States but is permitted in many other countries. Thus, it was decided to include in the collaborative study PG, BHA, and BHT which are permitted in Canada and the United States, as well as THBP, TBHQ, and Ionox-100 which are permitted in the United States but not in Canada. NDGA, a naturally occurring antioxidant formerly permitted in the United States and Canada, was also included. Generally, when used, these antioxidants are permitted either singly or in combination in fats and oils at levels up to 200 ppm.

Collaborative Study

After initial studies to evaluate spiked sample stability and homogeneity, samples were prepared by adding appropriate aliquots of each antioxidant in isopropanol to tared round-bottom flasks containing degassed blank sunflower seed oil or liquified lard. The isopropanol was removed under vacuum with gentle heat and stirring. Individual aliquots, ca 6 g oil or 3 g lard, were removed, sealed in Teflon-lined screw-cap vials under nitrogen, and stored at −20°C. In total, 10 different oil and 4 different lard samples were prepared.

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The recommendation of the Associate Referee was approved by the General Referee and Committee C and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, 363; 419.

In oils, 3 spiking levels were prepared: ca 200 ppm, to evaluate the method's performance for single antioxidant use near the regulatory level, and ca 100 and 20 ppm to evaluate the method for combined antioxidants or lower levels of use. NDGA was spiked at ca 100 and 20 ppm. For lards, antioxidants were added at ca 100 and 40 ppm. Thus, each oil sample contained 4 or 5 antioxidants and would be blank for the others, whereas each lard sample contained all 7 antioxidants with no blanks.

The samples prepared consisted of randomly scattered closely matched pairs according to the collaborative test described by Youden (2). They were spiked at 3 levels in oil (3 pairs and 4 blanks in 10 samples) and at 2 levels in lard (2 pairs with no blanks in 4 samples) except for NDGA which was spiked at only 2 levels in oil (2 pairs and 6 blanks in 10 samples).

The collaborators were supplied with the 14 spiked samples, a copy of the method, antioxidant standards, a blank oil for practice recovery studies, instructions for the analysis of the samples, and forms for reporting results and instrumental parameters. They were asked to run a blank gradient, to carry out a reagent blank determination, to carry out a blank oil determination, and finally to carry out a practice recovery study at 20 ppm. If recoveries >90% for all antioxidants except BHT and >80% for BHT were obtained, they were to proceed with the analysis of the 14 samples.

The collaborators were instructed to extract and concentrate the sample and to make only one injection of each sample, to dilute the sample as necessary and re-inject, and to alternate standard and sample injections. Enough sample was supplied for only one extraction. The calculated antioxidant level, the average standard peak heights (before and after sample), the sample peak heights, and dilution factors where applicable, were to be recorded on the appropriate report form. In addition, collaborators were asked to furnish copies of the chromatograms obtained and to list the chromatographic conditions and instrumentation.

Thirteen collaborators agreed to participate in the study and in August 1979 all samples were prepared and 8 collaborators were sent samples and instructions. In November 1979, samples were sent to an additional 3 collaborators, and in March 1980 and in March 1981 additional collaborators were sent samples. Analyses of the samples by the Associate Referee at this latest date showed no apparent degradation of the samples. In total, 8 collaborators reported results.

In general, the collaborators analyzed the spiked samples using the method previously reported by the Associate Referee (1) with the following exceptions: Only enough sample was provided for one determination (extraction); therefore, the sample was weighed in a tared 50 mL beaker and transferred with repeated rinsings to the separatory funnel before extraction.

In preliminary discussions with potential collaborators it became apparent that some analysts were having difficulty in acquiring the desired chromatographic column with the specified 3.2 mm id. Therefore, collaborators were permitted to use the more readily available 4.6 mm id column with 2 mL/min flow rate.

Shortly after the first samples were sent, one of the collaborators reported extremely low recoveries for TBHQ in the practice recovery study. Good recoveries were obtained for the other antioxidants. After some discussion and experimentation, it was found that the low recoveries for TBHQ, presumably due to oxidation, were occurring during the 30 min period taken to evaporate the acetonitrile sample extract, using the specified 40°C water bath. This evaporation, with quantitative recovery, could be carried out in 8 min in the Associate Referee's laboratory. The loss of TBHQ could be avoided if the extract were evaporated in less than 10 min. All collaborators were alerted to this requirement before any sample analyses were begun.

Antioxidants in Oil

Liquid Chromatographic Method

Official First Action

IUPAC-AOAC Method

Applicable to propyl gallate (PG), 2,4,5-trihydroxybutyrophenone (THBP), *tert*-butylhydroquinone (TBHQ), nordihydroguaiaretic acid (NDGA), 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT).

20.D01 ***Principle***

Sample is dissolved in hexane and antioxidants are extd into acetonitrile. Soln is concd, dild with equal vol. isopropanol, and injected into liq. chromatograph with UV detection at 280 nm.

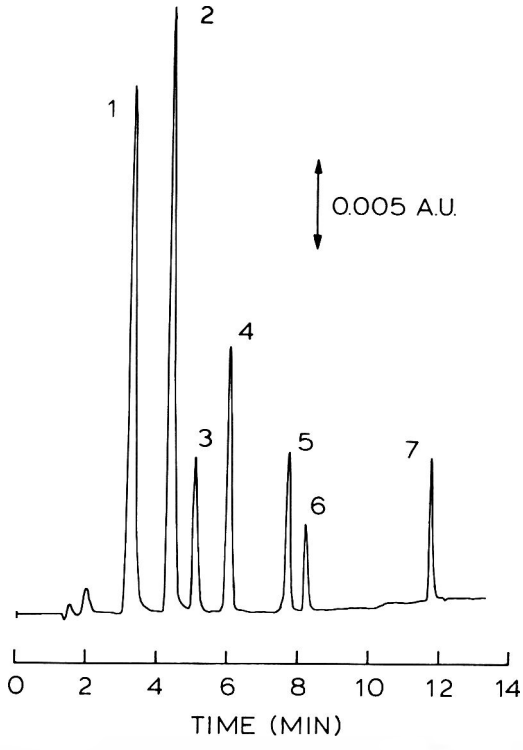

Fig. 20:D1. Chromatographic separation of antioxidant standards, ca 0.2 μg each antioxidant: 1, PG; 2, THBP; 3, TBHQ; 4, NDGA; 5, BHA; 6, Ionox-100; 7, BHT.

20.D02 *Apparatus and Reagents*

(a) *Gradient liquid chromatograph.*—Equipped with 10 mV strip chart recorder, 20 μL sample loop injection valve, and detector to measure *A* at 280 nm. Typical operating conditions: detector sensitivity, 0.05 AUFS; time const, 0; temp., ambient; flow rate, 2 mL/min.

(b) *Chromatographic column.*—Stainless steel, 250 × 4.6 mm id packed with 10 μm LiChrosorb RP-18 (E. Merck, Darmstadt, GFR), or equiv. Use guard column if desired. Baseline separation of all 7 antioxidants should be obtained as shown in Figure **20:D1.**

(c) *Glassware.*—Rinse all glassware with $CHCl_3$, acetone, and MeOH, in that order, and blow dry with N.

(d) *Solvents.*—Distd-in-glass acetonitrile, 2-propanol, and hexane.

(e) *Mobile phase.*—Use HPLC grade solvs, or equiv. *A. Distilled H_2O.*—Add 5% HOAc. *B. Acetonitrile.*—Add 5% HOAc. Use linear gradient, from 30% B in A to 100% B over 10 min with 4 min hold at 100% B at 2 mL/min. For sample only, increase flow rate to 6 mL/min at 100% B for 5 min or until nonpolar lipids are eluted. For samples and stds, return to 30% B in A over 1 min at 2 mL/min and let baseline, pressure, and mobile phase composition stabilize (ca 10 min). Run blank gradient (no injection). Peaks interfering with detn of any antioxidant should not be present. If small peaks are present which cannot be eliminated, all relevant peak heights are to be corrected for interference.

(f) *Antioxidants.*—BHA (mixt. of 2- and 3-BHA), BHT, TBHQ, Ionox-100, THBP, and PG (available from Polyscience Corp., Niles, IL 60648); NDGA (Food Chemicals Codex Ref. Std), or equiv.

(g) *Std solns.*—Refrigerate all antioxidant solns out of direct light. Prep. all solns with 2-propanol–acetonitrile (1 + 1). (*1*) *Stock soln.*—1 mg/mL. Accurately weigh and transfer 50 mg each antioxidant into one 50 mL vol. flask, dissolve, dil. to vol., and mix. (*2*) *Std soln.*—0.01 mg/mL (10 μg/mL). Pipet 1 mL stock soln into 100 mL vol. flask, dil. to vol., and mix.

(h) *Extracting solvents.*—Sat. hexane and acetonitrile by shaking together 2 min, and sep. Unless otherwise specified, use these satd solvs in *Extraction* below.

20.D03 ***Determination***

(a) *Extraction.*—(*1*) *Liquid oils.*—Accurately weigh ca 20 g oil into 50 mL beaker and quant. transfer to 100 mL vol. flask, rinsing beaker with hexane. Dil. to vol. with hexane and mix. Pipet 25 mL aliquot into 125 mL separator and ext with three 50 mL portions of acetonitrile. If emulsions form, break by holding separator under hot tap H_2O 5–10 s. Collect exts in 250 mL separator and let combined exts slowly drain into 250 mL r-b flask to aid removal of hexane–oil droplets. Note: At this point, the 150 mL acetonitrile ext may be stored overnight under refrigeration.

Evap. to 3–4 mL, using flash evaporator with ≤40° H_2O bath and completing evapn in <10 min. Note: Losses of TBHQ may occur if evapn time is prolonged. Use efficient vac. source and ice–H_2O cooling to decrease evapn time. Using disposable pipet, transfer acetonitrile–oil droplet mixt. to 10 mL g-s graduate. Rinse flask with small portions of non-satd acetonitrile and transfer rinsings to graduate with disposable pipet until 5 mL is collected. Rinse disposable pipet and continue to rinse flask with small portions of 2-propanol, transferring all rinsings to graduate until exactly 10 mL is collected. Mix contents of graduate. Note: Avoid delay in analysis after prepg sample soln because loss of TBHQ may occur.

(*2*) *Lards or shortenings.*—Accurately weigh 10 g lard or shortening into 150 mL beaker. Add ca 30 mL hexane and dissolve sample, heating gently if necessary. Quant. transfer to 100 mL vol. flask, rinsing beaker with hexane. Dil. to vol. and mix. Continue extns as in (*1*) above, beginning "Pipet 25 mL aliquot . . .".

(b) *Chromatography.*—Using sample loop injection valve, inject, in duplicate, 20 μL prepd sample soln onto column, and solv. program as described. Inject 20 μL antioxidant std soln (10 μg/mL) and solv. program as described before and after each sample. For sample peaks off scale, quant. dil. sample soln with 2-propanol–acetonitrile (1 + 1). Identify peaks by comparison with retention times of std. Note: Octyl gallate (OG, available from Pfaltz and Bauer, Inc., Stamford, CT), if present, may co-elute with Ionox-100, but can be sepd with H_2O–MeOH gradient as follows: 30% B (MeOH with 5% HOAc) in A (H_2O with 5% HOAc) to 100% B over 10 min. If both Ionox-100 and OG are present, accurate quant. may not be possible.

Carry out reagent blank detn, substituting 25 mL hexane for hexane–oil. Continue extn as in (*1*) above, beginning "Pipet 25 mL aliquot . . ." Inject 20 μL reagent blank soln and solv. program as described. Peaks interfering with detn of any antioxidant should not be present. Using blank gradient chromatogram as guide to follow baseline, det. av. peak ht of antioxidant sample from duplicate injections (corrected for reagent and gradient blanks), and av. peak ht of antioxidant std from duplicate injections before and after sample (corrected for gradient blank).

20.D04 ***Calculation***

Calc. concn of antioxidant as follows:

$$\text{Antioxidant, ppm} = (C_s/R_s) \times (R_x/W_x) \times D$$

where R_x and R_s = peak hts from sample and std, resp.; C_s = concn of std in μg/mL; W_x = wt of sample in g/mL in 10 mL final ext; and D = diln factor if soln injected is dild.

Results

Results were reported by 8 collaborators. Collaborator 2 submitted only the form reporting the level of antioxidant found and the equipment used. All other collaborators submitted the requested chromatograms and completed reporting forms. Of the 5 collaborators who did not report results or analyze samples, one reported preliminary recovery values but due to staffing problems was unable to complete the study. The 4 other collaborators reported unavailability of equipment, change of employment, or lack of sufficient time as reasons for not analyzing the samples.

Statistical Evaluation

The results submitted by the 8 collaborators were subjected to ranking tests, 2-sample plots of the matched pairs, and Dixon's test for outliers as decribed by Youden (2) before further statistical treatment. From a visual inspection of the individual results from each collaborator as well as the statistical and graphical procedures described above, it was immediately apparent that Collaborator 2 had considerable difficulty with the procedure. Unfortunately, this collaborator did not submit the requested chromatograms or peak height measurements and, therefore, no reasons for the method's poor performance are known. The collaborator did, however, report difficulties in separating the pairs PG/THBP and BHA/Ionox-100. For Collaborator 2, 66.2% (45 of 68) of the values reported were outlying by Dixon's test (95% confidence level) or not calculable because of poor separation. For the other

Table 1. Distribution of outliers by Dixon's test (95% confidence level) and ranking test

Antioxidant	Collaborator 1	3	4	5	6	7	8	Total each antioxidant
PG	1[a]			1				2
THBP						4(H)[b]		4(H)
TBHQ			(L)[b]	3(H)	(L)			3(L)(H)(L)
NDGA	1			1				2
BHA				(H)	1			1(H)
Ionox-100		1		(H)		3		4(H)
BHT			4			1	1	6
Coll.	2	1	4(L)	5(H)	1(L)	8(H)	1	22
Total				(H)				
				(H)				

[a] Numbers refer to number of statistically outlying results by Dixon's test.
[b] Bracketed letters refer to high (H), or low (L) ranking test for outliers (95% confidence level).

7 laboratories, only 2.3% of the reported results were outlying by Dixon's test. Therefore, all the results from Collaborator 2 were rejected, and the ranking tests, 2-sample plots of the matched pairs, and Dixon's test were repeated for the remaining 7 laboratories. Dixon's test revealed 4.6% outlying results, with the ranking tests and the 2-sample plots generally confirming the outlying results. The distribution of outliers determined by the ranking test and Dixon's test, not including Collaborator 2, is shown in Table 1.

The statistical results for the study are separated by antioxidant with the oils in Tables 2–8 and the lard samples in Tables 9–15. The statistical results are summarized in Table 16. The outlying collaborative results by Dixon's test (95% confidence level) were not included in the statistical treatment. The values for s_d were calculated on 2 sets of data. The first was calculated from the individual results for each sample for each antioxidant and the second from the matched sample pairs. The CV (%) was calculated on the mean for the individual results and on one-half the mean for the matched sample pair totals. If one of the matched sample pair values was rejected, the other value of the pair, if not outlying, was included in the determination of s_d for the individual values.

The values for s_d, s_r, and s_b were calculated according to Youden (2). The *F*-test, for indication of systematic errors at the 90, 95, and 99% confidence levels, was calculated as described by Youden (2). The negative values for s_b are interpreted as lack of evidence for systematic error and support the *F*-test. Individual recoveries were not tabulated, but the average percent recovery (mean/added) is given.

Collaborators' Comments

The collaborator's instrumentation and conditions of analysis are summarized in Table 17. Further comments by the collaborators are given below.

Collaborator 1 experienced initial problems of poor peak shape and resolution which were rectified by using a 25 μL injection (rather than 50 μL), 0.05 AUFS (rather than 0.1), and a 20% acetonitrile in water to 100% acetonitrile gradient (both solvents with 5% acetic acid) rather than the 30 to 100% gradient specified. The acetonitrile extract was evaporated in 10 min on a 45°C water bath (40°C specified).

Collaborator 3 had problems obtaining a blank gradient free of interfering peaks. The water was found to be the cause and, after considerable experimentation, an acceptable source was finally found. The acetonitrile extract was evaporated in 10 min with a vacuum pump as the vacuum source.

Collaborator 4 reported a peak in the blank gradient corresponding to about 5.5 ppm BHT. This interference was *not* taken into consideration in calculating the peak heights. Recoveries of 84% were obtained for TBHQ in the preliminary recovery trial. The collaborator was unable to evaporate the acetonitrile solution in the required 10 min; 11–12 were required.

Collaborator 5 also had an interference with BHT in the blank gradient. This was eliminated by using acetic acid from another manufacturer. Even with this product, the 5% acetic acid-in-water eluant had to be prepared fresh daily to avoid the interference. Collaborator 5 also reported that a 25 μL injection gave distorted peaks and that a 10 μL injection was needed for satisfactory peak shape.

Table 2. Collaborative results for determination of PG (ppm) in oils[a]

	Sample				Sample				Sample			
Coll.	2	3	Diff.	Total	8	9	Diff.	Total	5	10	Diff.	Total
1	198	131[b]	—	—	98	88	10	186	11	17	−6	28
2	105[b]	166	—	—	61[b]	41.5[b]	—	—	8.5	7.0[b]	—	—
3	193.0	189.7	3.3	382.7	106.4	89.1	1	195.5	18.6	17.8	0.7	36.5
4	183.1	178.5	4.6	361.6	91.7	93.6	−1.9	185.3	16.5	17.2	−0.7	33.7
5	206	179	27	385	101	93.4	7.6	194.4	21.8	20.3[c]	—	—
6	200	173.2	26.8	373.2	97.9	92.8	5.1	190.7	17.8	17.1	0.7	34.9
7	191	182	9	373	94	86	8	180	19	17	2	36
8	193.1	184.4	8.7	377.5	91.1	90.8	0.3	181.9	18.7	16.5	2.2	35.2
Mean	194.9	181.1		375.5	97.2	90.5		187.7	17.6	17.1		34.15
Added	201.2	186.1			100.6	93.1			20.1	18.6		
Rec., %	96.9	97.3			96.6	97.2			87.7	92.0		
s_d	7.30	5.64		5.92	5.60	3.25		4.24	3.33	0.454		2.20
CV, %	3.75	3.11		3.16	5.77	3.59		4.52	18.9	2.65		12.95
s_r			7.65				4.50				2.15	
CV, %			4.01				2.40				12.6	
s_b			−ve				−ve				0.35	
CV, %											2.06	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.

Table 3. Collaborative results for determination of THBP (ppm) in oils [a]

	Sample				Sample				Sample			
Coll.	4	3	Diff.	Total	6	9	Diff.	Total	1	8	Diff.	Total
1	201	129	72	330	104	91	13	195	18	20	−2	38
2	NC[b]	126	—	—	56.5[c]	12[c]	—	—	2.5[c]	41.5[c]	—	—
3	212.9	198.0	14.9	410.9	107.8	95.3	12.5	203.1	19.5	19.3	0.2	38.8
4	209.6	184.0	25.6	393.6	99.9	95.7	4.2	195.6	18.9	18.1	0.8	37.0
5	217	182	35	399	108	100	8	208	21.6	20.3	1.3	41.9
6	209.5	179.0	30.5	388.5	93.6	88.7	4.9	182.3	18.7	17.7	1.0	36.4
7	255[c,d]	237	—	—	98	105	−7	203	21	23	−2	44
8	200.8	201.1	−0.3	401.9	98.6	101.2	−2.6	199.8	19.4	19.5	−0.1	38.9
Mean	208.5	187.2		387.3	101.4	96.7		198.1	19.6	19.7		39.3
Added	210.0	196.4			105.0	98.2			21.0	19.6		
Rec., %	99.2	95.3			96.6	98.4			93.3	100.5		
s_d	6.47	32.4		20.57	5.38	5.77		5.88	1.28	1.73		1.93
CV, %	3.10	17.3		10.6	5.31	5.96		5.94	6.55	8.82		9.82
s_r			17.2				5.25				0.970[e]	
CV, %			8.88				5.30				4.94	
s_b			7.99				1.87				1.18	
CV, %			4.13				1.89				6.01	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Not calculable; bad separation.
[c] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[d] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.
[e] Bias significant (90% confidence level).

Table 4. Collaborative results for determination of TBHQ (ppm) in oils [a]

	Sample				Sample				Sample			
Coll.	4	2	Diff.	Total	7	8	Diff.	Total	10	5	Diff.	Total
1	204	196	8	400	111	98	13	209	21	24	−3	45
2	39[b]	43.5[b]	—	—	ND[c]	21.5	—	—	1.5[b]	ND	—	—
3	210.7	185.8	24.9	396.5	107.4	92.2	15.2	199.6	19.4[d]	18.2[d]	1.2	37.6
4	174.1	171.9	2.2	346.0	84.6	58.7	25.9	143.3	15.8	17.8	−2.0	33.6
5	251	228[b,e]	—	—	148[b,e]	137[b,e]	—	—	21.4	23.0	−1.6	44.4
6[f]	197.9	179.6	18.3	377.5	104.4	87.7	16.7	192.1	15.5	13.3	2.2	28.8
7	238	199	39	437	101	90	11	191	21	21	0	42
8	193.6	183.3	10.3	376.9	98.1	84.2	13.9	182.3	19.2	16.9	2.3	36.1
Mean	209.2	185.9		389.0	101.1	85.1		186.2	19.04	19.2		38.2
Added	204.8	187.4			102.4	93.7			20.5	18.7		
Rec., %	102.9	99.2			98.7	90.9			92.9	102.5		
s_d	26.5	10.2		21.5	9.27	13.7		16.2	2.46	3.74		4.22
CV, %	12.6	5.46		11.03	9.17	16.2		17.3	12.9	19.5		22.1
s_r			9.45[g]				3.71[h]				1.50[g]	
CV, %			4.86				3.98				7.85	
s_b			13.6[g]				11.1[h]				2.79[g]	
CV, %			7.01				11.9				14.6	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Not detected.
[d] Error in calculation, corrected by Associate Referee.
[e] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.
[f] Baseline redrawn by Associate Referee, results recalculated.
[g] Bias significant (95% confidence level).
[h] Bias significant (99% confidence level).

Table 5. Collaborative results for determination of NDGA (ppm) in oils [a]

	Sample				Sample			
Coll.	6	9	Diff.	Total	4	3	Diff.	Total
1	92	84	8	176	19	17	2	36
2	24.5[b]	24.5[b]	—	—	20.5	27.5[b]	—	—
3	96.0	85.5	10.5	181.5	18.7	17.6	1.1	36.3
4	91.3	87	4.3	178.3	18.8	17.3	1.5	36.1
5	110[c]	95.7	—	—	19.1	17.1	2.0	36.2
6	82.9	91.0	−8.1	173.9	18.9	18.1	0.8	37.0
7	96	88	8	184	20	19	1	39
8	94.0	90.9	3.1	184.9	19.6	18.0	1.6	37.6
Mean	92.0	88.9		179.8	19.2	17.7		36.9
Added	97.7	89.9			19.5	18.0		
Rec., %	94.1	98.9			98.2	98.5		
s_d	4.88	3.97		3.13	0.472	0.702		0.775
CV, %	5.31	4.47		3.48	2.46	3.96		4.20
s_r			4.70				0.338[d]	
CV, %			2.62				1.83	
s_b			−ve				0.493	
CV, %							2.67	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.
[d] Bias significant (95% confidence level).

Collaborator 6, who noted the problem of TBHQ loss during evaporation, apparently still had difficulty in evaporating the acetonitrile extract in the specified 10 min.

Collaborators 2, 7, and 8 did not provide specific comments on the method.

Discussion

Statistical Results

The recoveries for each antioxidant given in Tables 2–15 are slightly higher for oil than for lard samples and also higher at the higher spiking levels than the lower ones for both oil and lard. Except for BHT, in which the expected 84–86% recoveries were obtained (1), average recoveries for all antioxidants, averaged over all spiking levels for oils and lards (Table 16), were relatively consistent, ranging from 93.2% for PG to 98.3% for BHA. The average recovery for TBHQ, in which only 3 high outlying results were excluded from the statistical calculation out of the 70 reported values, is quite good. However, this is due to a balance between Collaborators 4 and 6 which ranked as low outliers and Collaborator 5 which ranked high. These are shown in Table 1. These high and low outliers by the ranking tests were included in the statistical calculations and reflect the high coefficient of variation (CV, %) determined for TBHQ. The average random or within-laboratory component of the standard deviation, s_r, for TBHQ is not much greater than that for the other antioxidants. The average systematic or between-laboratory component s_b, however, is at least 4 times greater than that of the next highest antioxidant and twice the random component. This dominance of the systematic component is to be expected, considering the ranking results for Collaborators 4, 5, and 6 described above. The results for the *F*-test in Table 16 confirm this as 3 sample pairs show significant systematic error at the 95% confidence level and 2 pairs at the 99% level.

For PG, the s_b component of s_d was negative in 4 of the 5 sample pairs, indicating no evidence for between-laboratory (systematic) error. Similarly the *F*-test indicated no significant systematic error. This is reflected in the lack of outliers by ranking tests for PG as shown in Table 1. For THBP, there were no negative values for s_b and the *F*-test revealed no significant systematic error. Collaborator 7, which ranked as a high outlier, had 4 high results rejected by Dixon's test. With these outlying values omitted from the statistical calculations, significant systematic error was reduced. NDGA had a negative s_b in one of the 4 sample pairs and significant systematic error at the 95% confidence level (*F*-test) for 2 sample pairs. For NDGA the s_b term is greater than the s_r component. For BHA, one pair had a negative s_b and one pair had significant systematic error (95% confidence level).

Table 6. Collaborative results for determination of BHA (ppm) in oils [a]

	Sample				Sample				Sample			
Coll.	1	2	Diff.	Total	10	8	Diff.	Total	7	3	Diff.	Total
1	199	187	12	386	100	93	7	193	19	18	1	37
2	47[b]	128[b]	—	—	27[b]	218.5[b]	—	—	11[b]	22.5	—	—
3	213.6	180.6	33	394.2	103.2	99	4.2	202.2	20.1	18	2.1	38.1
4	204.4	190.7	13.7	395.1	101.5	97.8	3.7	199.3	19.8	19.1	0.7	38.9
5	216	197	19	413	101	105	−4	206	21.6	20.0	1.6	41.6
6	200.7	192.2	8.5	392.9	118.5[c]	97.2	—	—	20.8	18.5	2.3	39.3
7	196	191	5	387	98	92	6	190	20	19	1	39
8	198.3	188	10.3	386.3	99.5	89.5	10	189.0	19.9	18.7	1.2	38.6
Mean	204.0	189.5		393.5	100.5	96.2		196.6	20.2	18.8		38.9
Added	206.7	190.2			103.3	95.1			20.7	19.0		
Rec., %	98.7	99.6			97.3	101.2			97.7	98.7		
s_d	7.84	5.08		6.67	1.79	5.19		4.91	0.776	0.700		0.991
CV, %	3.84	2.68		3.39	1.78	5.39		5.00	3.84	3.73		5.09
s_r			6.54				3.34				0.427[d]	
CV, %			3.32				3.40				2.19	
s_b			0.929				2.63				0.633	
CV, %			0.471				2.67				3.25	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.
[d] Bias significant (95% confidence level).

Table 7. Collaborative results for determination of Ionox-100 (ppm) in oils [a]

	Sample				Sample				Sample			
Coll.	5	1	Diff.	Total	7	10	Diff.	Total	6	2	Diff.	Total
1	212	192	20	404	94	97	−3	191	20	19	1	39
2	355.0[b]	35.5[b]	—	—	77	36.5[b]	—	—	36.5[b]	ND[b,c]	—	—
3	215.4	165.7	49.7	381.1	103.5	82.0[d]	—	—	18.7	18.9	−0.2	37.6
4	209.8	188.9	20.9	398.7	98.9	94.5	4.4	193.4	20.4	19.6	0.8	40.0
5	228	207	21	435	115	99.1	15.9	214.1	22.5	20.4	2.1	42.9
6[e]	205.4	209.8	−4.4	415.2	116.8	96.5	20.3	213.3	20.0	20.1	−0.1	40.1
7	156[b,d]	185	—	—	78	97	−19	175	16	ND[b,d]	—	—
8	214.6	187.7	26.9	402.3	109	100.2	8.8	209.2	22.3	20.2	2.1	42.5
Mean	212.5	190.9		406.5	102.2	97.4		199.3	20.0	19.7		40.4
Added	216.9	199.4			108.4	99.7			21.7	19.9		
Rec., %	98.0	95.7			94.3	97.7			92.1	99.0		
s_d	8.40	14.7		12.7	13.5	2.01		10.99	2.21	0.639		1.44
CV, %	3.95	7.72		6.26	13.2	2.07		11.02	11.1	3.24		7.13
s_r			12.2				10.0				0.714[f]	
CV, %			6.02				10.1				3.54	
s_b			2.48				3.17				0.883	
CV, %			1.22				3.18				4.38	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Not detected.
[d] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.
[e] Baseline redrawn by Associate Referee, results recalculated.
[f] Bias significant (90% confidence level).

Table 8. Collaborative results for determination of BHT (ppm) in oils [a]

	Sample				Sample				Sample			
Coll.	5	4	Diff.	Total	7	6	Diff.	Total	1	9	Diff.	Total
1	164	163	1	327	90	80	10	170	18	16	2	34
2	433.0[b]	153	—	—	56.0	ND[b,c]	—	—	ND[b,c]	8.0[b]	—	—
3	170.0	171.6	−1.6	341.6	85.9	83.6	2.3	169.5	20.1	17.1	3.3	37.5
4	133.5[d]	55.8[b,d]	—	—	69.5[d]	63.5[b,d]	—	—	18.6	16.5	2.1	35.1
5	171	165	6	336	84.2	80.5	3.7	164.7	17.8	17.0	0.8	34.8
6	175.6	172.4	3.2	348.0	89.4	78.7	10.7	168.1	16.8	17.2	−0.4	34.0
7	174	169	5	343	88	82	6	170	18	16	2	34
8	179.1	166.3	12.8	345.4	88	81.2	6.8	169.2	17.4	14.4[d]	—	—
Mean	172.3	167.9		340.2	87.6	81.0		168.6	18.1	16.6		34.9
Added	210.4	195.4			105.2	97.7			21.0	19.5		
Rec., %	81.9	85.9			83.3	82.9			86.4	85.3		
s_d	5.21	3.75		5.38	2.18	1.69		1.43	1.14	0.547		0.961
CV, %	3.03	2.23		3.16	2.49	2.09		1.70	6.29	3.29		5.51
s_r			3.50				2.36				0.900	
CV, %			2.06				2.80				5.16	
s_b			2.89				−ve				0.239	
CV, %			1.70								1.37	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Not detected.
[d] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.

Table 9. Collaborative results for determination of PG (ppm) in lard [a]

	Sample				Sample			
Coll.	14	11	Diff.	Total	12	13	Diff.	Total
1	93	84	9	177	36	33	3	69
2	76[b]	70[b]	—	—	44[b]	20.5[b]	—	—
3	101.4	83.2	18.2	184.6	35	34.2	0.8	69.2
4	99.4	85.1	14.3	184.5	38.2	31.0	7.2	69.2
5	96.0	85.3	10.7	181.3	38.3	31.8	6.5	70.1
6	93.0	86.3	6.7	179.3	37.7	33.2	4.5	70.9
7	90	85	5	175	35	32	3	67
8	94.8	84.9	9.9	179.7	36.4	32.5	3.9	68.9
Mean	95.4	84.8		180.2	36.7	32.5		69.9
Added	100.6	93.1			40.2	37.2		
Rec., %	94.8	91.1			91.2	87.4		
s_d	3.95	0.986		2.54	1.42	1.05		0.850
CV, %	4.14	1.16		2.82	3.88	3.22		2.43
s_r			3.18				1.55	
CV, %			3.53				4.44	
s_b			−ve				−ve	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).

The systematic error arises from Collaborator 5, which ranked as a high outlier yet was not excluded on the basis of Dixon's test and was therefore included in the statistical calculations. For Ionox-100 and BHT, the *F*-test did not demonstrate significant systematic error.

For TBHQ, the systematic component (s_b) of the standard deviation dominated the precision component (s_r) whereas for the other antioxidants, s_r was greater for 5 antioxidants and slightly less for one. Possible explanations for these statistical results are given in the following section.

Sources of Error

Variations in the method, whether avoidable or inherent, can result in low or high recoveries

Table 10. Collaborative results for determination of THBP (ppm) in lard [a]

	Sample				Sample			
Coll.	12	14	Diff.	Total	11	13	Diff.	Total
1	97	91	6	188	37	35	2	72
2	79.5	44[b]	—	—	45.5	28.5	—	—
3	101.6	98.4	3.2	200.0	39.1	37.0	2.1	76.1
4	92.8	96.2	−3.4	189.0	35.0	34.7	0.3	69.7
5	105	97.1	7.9	202.1	38.7	35.1	3.6	73.8
6	94.7	77.6	17.1	172.3	38.4	32.8	5.6	71.2
7	124	126[b,c]	—	—	48[b,c]	51[b,c]	—	—
8	78.4	91.3	−12.9	169.7	39.0	34.6	4.4	73.6
Mean	99.1	91.9		186.9	37.9	34.9		72.73
Added	105	98.2			42.0	39.3		
Rec., %	94.4	93.6			90.2	88.7		
s_d	13.8	7.66		9.58	1.60	1.34		1.59
CV, %	14.0	8.33		10.3	4.22	3.85		4.37
s_r			7.25				1.35	
CV, %			7.76				3.71	
s_b			4.43				0.597	
CV, %			4.73				1.64	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.

Table 11. Collaborative results for determination of TBHQ (ppm) in lard [a]

	Sample				Sample			
Coll.	13	14	Diff.	Total	12	11	Diff.	Total
1	101	86	15	187	40	35	5	75
2	53.5	134	—	—	75	21	—	—
3	98.3[b]	94.2	4.1	192.5	36.8	33.1	3.7	69.9
4	86.5	73.2	13.3	159.7	23.1	17.1	6.0	40.2
5	134	125	9	259	41.8	43.9	−2.1	85.7
6[c]	84.6	47.2	37.4	131.8	14.6	29.8	−15.2	44.4
7	123	109	14	232	47	54	−7	101
8	91.9	84.4	7.5	176.3	41.0	32.1	8.9	73.1
Mean	102.8	88.4		191.2	34.9	33.3		69.9
Added	102.4	93.7			41.0	37.5		
Rec., %	100.3	94.4			85.1	88.7		
s_d	18.8	25.0		30.3	11.6	12.7		15.2
CV, %	18.3	28.3		31.7	33.3	38.2		43.6
s_r			7.71[d]				6.04[e]	
CV, %			8.04				17.3	
s_b			20.7				9.89	
CV, %			21.6				28.3	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Error in calculation, corrected by Associate Referee.
[c] Baseline redrawn by Associate Referee; results recalculated.
[d] Bias significant (99% confidence level).
[e] Bias significant (95% confidence level).

and increased within-laboratory (precision) and between-laboratory (systematic) components of the overall standard deviation. In this section, the probable causes of these variations in the method will be discussed and related to the results obtained by the collaborators.

As mentioned earlier, the loss of TBHQ during evaporation of the acetonitrile extract is probably

Table 12. Collaborative results for determination of NDGA (ppm) in lard [a]

	Sample				Sample			
Coll.	12	11	Diff.	Total	14	13	Diff.	Total
1	89	88	1	177	39	37[b]	—	—
2	135.0[c]	88	—	—	49[c]	62[c]	—	—
3	88.0	82.8	5.2	170.8	38.2	34.2[d]	4.0	72.4
4	88.9	81.6	7.3	170.5	33.9	33.0	0.9	66.9
5	97.7	92.7	5.0	190.4	37.2	32.5	4.7	69.7
6	87.6	75.2	12.4	162.8	35.6	32.1	3.5	67.7
7	93	85	8	178	37	32	5	69
8	94.9	84.6	10.3	179.5	38.6	34.3	4.3	72.9
Mean	91.3	84.3		175.6	37.1	33.0		69.8
Added	97.7	89.9			39.0	36.0		
Rec., %	93.4	93.7			95.1	91.7		
s_d	3.93	5.43		6.16	1.81	1.02		1.73
CV, %	4.30	6.45		7.01	4.87	3.09		4.95
s_r			2.65[e]				1.05	
CV, %			3.02				3.00	
s_b			3.93				0.970	
CV, %			4.48				2.78	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.
[c] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[d] Error in calculation, corrected by Associate Referee.
[e] Bias significant (95% confidence level).

Table 13. Collaborative results for determination of BHA (ppm) in lard [a]

	Sample				Sample			
Coll.	12	11	Diff.	Total	14	13	Diff.	Total
1	95[b,c]	93	2	188	39	39	0	78
2	100.5	97	—	—	50[b]	49.5[b]	—	—
3	100	89.0	11	189	39.0	36.4	2.6	75.4
4	101.9	92.8	9.1	194.7	39.6	38.6	1.0	78.2
5	107[b,c]	101	6	208	41.6	37.9	3.7	79.5
6	101.0	93.9	7.1	194.9	41.7	33.4	8.3	75.1
7	101	95	6	196	40	36	4	76
8	102.9	90.4	12.5	193.3	38.2	36.1	2.1	74.3
Mean	101.3	93.6		194.8	39.9	36.8		76.6
Added	103.3	95.1			41.3	38.0		
Rec., %	98.0	98.4			96.5	96.8		
s_d	3.58	3.86		4.64	1.34	1.92		1.36
CV, %	3.54	4.12		4.76	3.35	5.22		3.55
s_r			2.49[d]				1.90	
CV, %			2.56				4.97	
s_b			2.76				−ve	
CV, %			2.84					

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] When results of Collaborator 2 are not included, results not outlying. Included in statistical calculations.
[d] Bias significant (90% confidence level).

due to oxidation. A rapid evaporation will reduce the temperature of the acetonitrile solution being evaporated and will reduce the time that TBHQ is subjected to the elevated temperature of the bath. In the Associate Referee's laboratory, TBHQ was the antioxidant most susceptible to oxidation after sample evaporation (1). Furthermore, TBHQ in standard solutions of iso-

Table 14. Collaborative results for determination of Ionox-100 (ppm) in lard [a]

	Sample				Sample			
Coll.	13	12	Diff.	Total	14	11	Diff.	Total
1	107	89	18	196	43	39	4	82
2	120.0	125.5[b]	—	—	58.5[b]	38.5	—	—
3	99.0	84.9[c]	14.1	183.9	40.8	35.3	5.5	76.1
4	101.3	96.9	4.4	198.2	42.3	39.4	2.9	81.7
5	110	100	10	210	42.2	37.4	4.8	79.6
6[d]	108.4	99.0	9.4	207.4	36.6	35.6	1.0	72.2
7	97	100	−3	197	45	54[b,e]	—	—
8	101.9	96.0	5.9	197.9	40.7	38.7	2.0	79.4
Mean	103.5	95.1		198.6	41.5	37.6		78.5
Added	108.4	99.7			43.4	39.9		
Rec., %	95.5	95.4			95.7	94.2		
s_d	4.97	5.90		6.01	2.61	1.77		2.64
CV, %	4.80	6.20		6.05	6.28	4.72		6.74
s_r			4.83				1.21[f]	
CV, %			4.87				3.09	
s_b			2.53				1.66	
CV, %			2.54				4.24	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Error in calculation; corrected by Associate Referee.
[d] Baseline redrawn by Associate Referee; results recalculated.
[e] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.
[f] Bias significant (90% confidence level)

Table 15. Collaborative results for the determination of BHT (ppm) in lard [a]

	Sample				Sample			
Coll.	11	13	Diff.	Total	12	14	Diff.	Total
1	91	90	1	181	34	34	0	68
2	133.5[b]	94.0	—	—	96[b]	36	—	—
3	93.2	87.2	6.0	180.4	35.4	35.2	0.2	70.6
4	88.5	73.3	15.2	161.8	35.6	34.3	1.3	69.9
5	93.1	87.4	5.7	180.5	37.3	33.6	3.7	70.9
6	88.4	82.3	6.1	170.7	36.3	34.5	1.8	70.8
7	100[c]	81	—	—	35	32	3	67
8	89.0	85.7	3.3	174.7	35.5	31.7	3.8	67.2
Mean	90.5	83.8		174.9	35.6	33.6		69.2
Added	105.2	97.7			42.1	39.1		
Rec., %	86.1	85.8			84.5	86.0		
s_d	2.23	5.58		5.37	1.03	1.30		1.23
CV, %	2.47	6.66		6.14	2.89	3.07		3.56
s_r			3.42				1.11	
CV, %			3.91				3.22	
s_b			2.93				0.370	
CV, %			3.35				1.07	

[a] All results from Collaborator 2 excluded from statistical calculations.
[b] Rejected as outlier (Dixon's test, 95% confidence level, all results including Collaborator 2).
[c] Rejected as outlier (Dixon's test, 95% confidence level, not including results of Collaborator 2). Omitted from statistical calculations.

Table 16. Statistical summary, average of 10 samples (8 for NDGA) as 5 sample pairs from Tables 2–15 (outliers omitted)

		Coefficient of variation, %				*F*-test[c]	
Antioxidant	Rec., %	s_d[a]	s_d[b]	s_r	s_b	95%	99%
PG	93.2	5.02	5.18	5.40	2.06 (1)[d]	0	0
THBP	95.1	7.74	8.21	6.12	3.68 (5)	0	0
TBHQ	95.6	19.3	25.1	8.41	16.7 (5)	3	2
NDGA	95.5	4.36	4.91	2.62	3.31 (3)	2	0
BHA	98.3	3.75	4.36	3.29	2.31 (4)	1	0
Ionox-100	95.8	6.33	7.43	5.52	3.11 (5)	0	0
BHT	84.8	3.45	4.01	3.43	1.87 (4)	0	0

[a] Calculated from individual results.
[b] Calculated from one half pair total and s_d.
[c] *F*-test for significant bias at 2 levels of confidence, number refers to number of sample pairs for which that level of bias exists.
[d] Bracketed number refers to number of values averaged to give figure, other values being negative.

Table 17. Instrumentation and conditions of analysis

Coll.	Instrument	Column	Flow, mL/min	Injection, μL
1	Waters Model 660	5 μm LiChrosorb RP-18	2.0	25
2	Varian Model 5000	10 μm LiChrosorb RP-18, 4.6 × 250 mm	2.0	10
3	Perkin-Elmer Series 3	10 μm LiChrosorb RP-18, 4.6 × 250 mm	2.0	20
4	Spectra-Physics Model SP8000	10 μm LiChrosorb RP-18, 4.6 × 250 mm	2.0	27.5
5	Spectra-Physics Model 3500 B	10 μm Zorbax ODS, 4.6 × 250 mm	1.2	10
6	Waters Model 660	10 μm Radial PAK A C_{18}, 8 × 100 mm	1	50
7	Waters Model 660	10 μm LiChrosorb RP-18, 3.2 × 250 mm	1	50
8	Altex-Beckman Model 420	10 μm LiChrosorb RP-18, 4.6 × 250 mm	2	20

propanol–acetonitrile (1 + 1) was the first antioxidant lost on standing. The decrease in the TBHQ peak was accompanied by the appearance of another peak just after NDGA. In the Associate Referee's laboratory, a water aspirator provides sufficient vacuum to evaporate the acetonitrile extract in 8–9 min. Ice water from an ice water bath is recycled through the condenser of the rotary evaporator. It may be advisable to carry out evaporations using this cooling procedure.

Several collaborators experienced problems with the blank (no injection) gradient. Two problems occurred. One involved a drift of the baseline and the other, more serious problem arose from extraneous or interfering peaks. Some chromatograms representative of these problems are presented in Figure 1. A sloping baseline such as those shown in Figure 1A and B, may give difficulties in the interpretation of the actual baseline to be used in determining peak heights. A blank gradient in which interfering peaks are present (Figure 1A) is a more serious problem.

These peaks generally arise from nonpolar impurities in the weaker elution solvent (5% acetic acid in water) accumulating at the head of the column before injection. These impurities are then eluted during the course of the gradient program. The peak size depends on the volume of weaker solvent pumped through the column before the gradient run commences. The current availability of HPLC grade solvents (water, acetonitrile, and acetic acid) allows this problem to be readily solved. Three collaborators reported problems with extraneous peaks. One collaborator found a peak to arise from the acetic acid and it was eliminated by using another brand. Another collaborator found a different source of water did not give rise to peaks. Collaborator 4 was unable to eliminate a peak corresponding to about 5.5 ppm BHT. This may be the cause for the outlying results. For sample and standard peaks of approximately the same height, the effect of the underlying false peak would effectively cancel. Thus, reasonable results were obtained by Collaborator 4 for pairs 1, 9 and 12, 14.

The method instructed the collaborator to run a blank gradient (no injection) and to use the chromatogram obtained as a guide to draw a baseline for peak height measurements. Two collaborators obtained poor results with this procedure. As shown in Figure 1C, with a drifting baseline it may be difficult to correctly interpret the correct peak height. Thus, with the

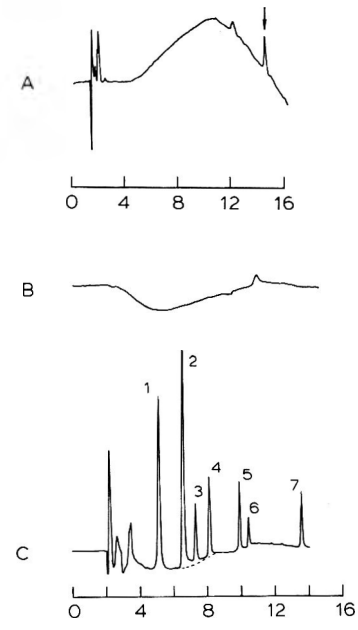

Figure 1. Collaborators' chromatograms illustrating: A, blank gradient with sloping baseline and interference with BHT; B, blank gradient with sloping baseline; C, standard solution with sloping baseline as in B, 1, PG; 2, THBP; 3, TBHQ; 4, NDGA; 5, BHA; 6, Ionox-100; 7, BHT; dotted line indicates probable baseline.

7 peaks present in the standard, a valley-to-valley baseline will result in an underestimate of the standard peak height. With only one peak present, especially with some of the diluted samples, the correct height will be measured. If the peak height of the sample is much greater than that of the standard, the effect is accentuated and high results can be obtained. In some instances for Collaborator 5, this may have accounted for the high outlying results for TBHQ.

Collaborator 6 also found difficulty in interpreting the baseline for TBHQ. For the standards as well as some samples, TBHQ appeared as a rider peak on the tailing THBP. Apparently with the Radial PAK C_{18} column, PG and THBP tail. This also may be due to too large an injection volume. The TBHQ was estimated from a flat baseline as shown in Figure 2A. With the concurrence of Collaborator 6, the Associate Referee re-estimated the TBHQ peak height as a skimmed-off rider peak on the tail of the THBP peak. The recalculated peaks are given in Tables 4 and 11. Collaborator 6 also had a broad peak arising from the blank gradient occurring under the Ionox-100 peak as shown in Figure 2A and B.

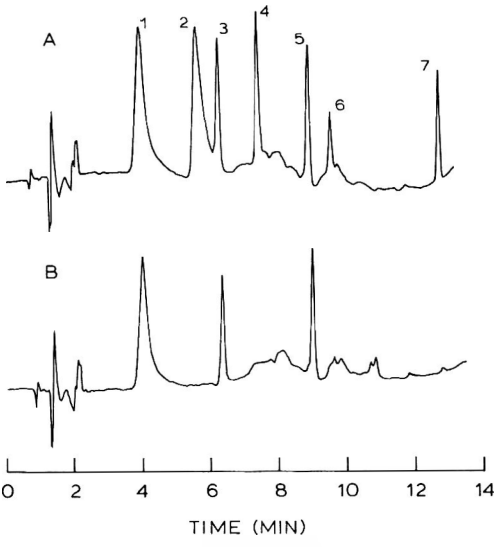

Figure 2. Collaborator's chromatograms showing: A, standard solution as in Fig. 1C with TBHQ as rider peak on THBP tail; B, broadened peaks under Ionox-100.

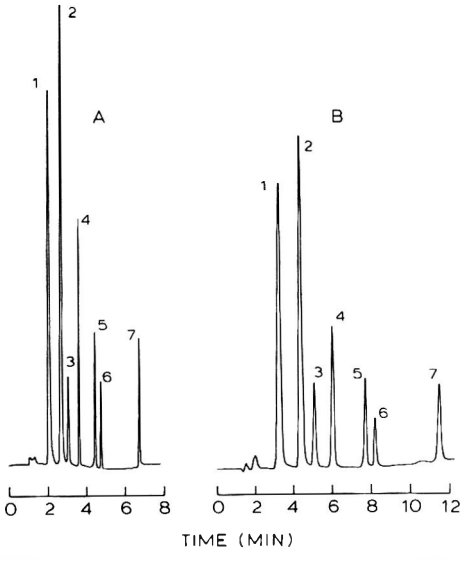

Figure 3. Chromatograms of standard solutions as in Fig. 1C: A, 5 μm 4.6 × 150 mm Supelcosil RP-18 with 30 to 100% B gradient over 5 min; B, collaborator's column, 4.6 × 250 mm 10 μm LiChrosorb RP-18.

Again with the concurrence of Collaborator 6, the peak height was remeasured and the Ionox-100 concentration was recalculated. These new values are included in Tables 7 and 14.

The problems in baseline interpretation may be very subtle as described above for Collaborator 5 with TBHQ or may be readily observed as with Collaborator 6.

One of the factors affecting column performance is the polarity of the sample solvent. In reverse phase chromatography, it is good chromatographic practice to dissolve samples in the mobile phase, or in a solvent miscible with the mobile phase but of similar or greater polarity, i.e., weaker than the eluting solvent. With the method studied this is not the case. To dissolve the co-extracted lipid material and to ensure dissolution of the nonpolar BHT, the isopropanol–acetonitrile (1 + 1) solvent is necessary. If too much of this solvent is injected onto the column, it acts as an eluting solvent and double or distorted peaks can result. This seems to occur to a lesser degree if a guard column is used. Several collaborators reduced their injection volume to improve the peak shape. Thus it is not known whether the poor column performance for Collaborator 7 resulted from the large volume injected (50 μL onto a 3.2 × 250 mm column) or from a deteriorated column. Some of the outlying results for Collaborator 7 definitely arise from the poor resolution obtained. In one instance, Ionox-100 at 19.9 ppm was not detected in the presence of 190.2 ppm BHA.

Most of this collaborative work was carried out in late 1979 or early 1980. Since that time, a greater availability and acceptance of HPLC grade solvents has occurred. This, coupled with the increasing experience of most analysts in gradient analysis, should reduce problems arising from serious baseline drift and extraneous interfering peaks.

Also in the last few years, the standard analytical column used by practicing chromatographers has changed to some extent. More analysts are using the 4.6 × 150 mm column packed with 5 μm spherical particles. In fact, the column size suggested for use in this study, 3.2 × 250 mm, is now seldom available. In the Associate Referee's laboratory, the 5 μm column, with an octadecyl silyl bonded phase, gives superior resolution and sensitivity as shown in Figure 3A. The column used by most collaborators, the 4.6 × 250 mm 10 μm LiChrosorb RP-18, however, still gives good chromatographic separations (Figure 3B). Thus, if the injected volume is specified to avoid solvent overload and the recently available 5 μm spherical particle columns are employed, column performance for all samples and antioxidants should be excellent. In addition, these shorter columns with the spher-

ical particles permit rapid re-equilibration with the use of higher flow rates with lower back pressure than the irregular shaped particle packings. Figure 3 demonstrates this.

The problem of TBHQ loss can be avoided during evaporation if the evaporation time is kept as short as possible (less than 10 min). This necessitates an efficient vacuum source and most probably the use of ice water in the condenser.

Considering the good results obtained for 6 of the 7 antioxidants, it is felt that comparable results should be obtained for TBHQ provided acceptable chromatographic conditions are maintained and losses on evaporation can be avoided as described above.

Recommendation

It is recommended that the method be adopted official first action for PG, THBP, TBHQ, NDGA, BHA, Ionox-100, and BHT in fats and oils.

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VITAMINS AND OTHER NUTRIENTS

Isomerization of Retinyl Palmitate Using Conventional Lipid Extraction Solvents

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The use of a chloroform-ethanol-water solvent system for the direct extraction of retinyl palmitate isomers from fortified food products was previously shown to be unsuitable because significant isomerization of all-*trans*-retinyl palmitate occurred during the extraction. This study investigated the extent of isomerization of retinyl palmitate in various extraction solvents when subjected to gold fluorescent laboratory light. Purified solutions of all-*trans*-retinyl palmitate in hexane were diluted with methyl *t*-butyl ether, hexane, methylene chloride, and stabilized chloroform and subjected to gold fluorescent laboratory light for 2, 4, and 6.5 h. Similar solutions were subjected to light or kept in the dark for 3.5 h. All-*trans*-, 9-*cis*-, and 13-*cis*-retinyl palmitate esters in the solutions were determined by using normal phase high performance liquid chromatography with fluorometric detection. Results demonstrated a noticeable increase in the 9-*cis*-retinyl palmitate concentration and a corresponding decrease in all-*trans*-retinyl palmitate concentration with time, in chloroform and methylene chloride compared with hexane. Chlorinated solvents in the absence of light did not promote isomerization of retinyl palmitate. Use of chlorinated solvents for the extraction of vitamin A esters should be avoided because they promote isomerization of retinyl palmitate when subjected to light, including gold fluorescent laboratory light.

Direct extraction of all-*trans*-retinyl palmitate from food products without saponification for subsequent high performance liquid chromatographic (HPLC) analysis has proven to be the method of choice by several laboratories (1–7). The chlorinated solvents chloroform and methylene chloride are often used either for the extraction of vitamin A palmitate from food products and/or as a component of the mobile phase (2–4, 6, 7). These methods are able to quantitate only all-*trans*-retinyl palmitate, the most biologically active form of vitamin A. Other isomers, such as the 9-*cis*- and 13-*cis*-retinyl palmitate have a biological activity of 26% and 75%, respectively, of all-*trans*-retinyl palmitate (8); therefore, the actual biological activity of vitamin A in fortified foods is overestimated if *cis*-isomers of retinyl esters are present. Thompson et al. (5) discussed the separation of the 13-*cis*-isomer from the all-*trans*-retinyl palmitate, using their HPLC method, but quantitation of the 13-*cis*-isomer in milk products was not performed.

Currently, several HPLC methods are able to separate the isomeric forms of the retinyl esters (9–12). None of these procedures, however, has been used for routine determination of vitamin A esters in food products.

An attempt was made in our laboratory to adapt the extraction procedure of Widicus and Kirk (2) to the determination of retinyl palmitate isomers in foods. The method proposed was a variation of the Bligh and Dyer (13) extraction procedure which specifies a mixture of chloroform–ethanol–water in place of the more popular chloroform–methanol–water solvent system. Analytical data demonstrated the unsuitability of having chloroform in the extraction solution because significant isomerization of the retinyl palmitate occurred during extraction. Isomerization was not observed during the storage of purified retinyl palmitate standards in hexane. Thus, chloroform appeared to be initiating the isomerization reaction, possibly by a free-radical mechanism.

It is well known that chloroform is unstable in the presence of light and oxygen, forming undesirable degradation products such as phosgene and HCl, which may initiate acid-catalyzed isomerization of unsaturated double bond systems. Thus, there was the possibility that acid-catalyzed isomerization was occurring during extraction (14). Addition of absolute ethanol (0.5–2.0%) or an unsaturated hydrocarbon (e.g., amylene) is thought to slow these reactions in chloroform. Treatment of chloroform with a molecular sieve (Type 13X) has been shown to remove water, HCl, and phosgene (15).

Solvent effects on the photo-isomerization of retinoid compounds has been observed previously by researchers (16, 17). These solvent-

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induced changes were observed under intense radiation, after long exposure times (up to 24 h) with or without the addition of iodine to catalyze the stereochemical rearrangement.

The purpose of this study was to investigate the extent of isomerization of all-*trans*-retinyl palmitate in various extraction solvents subjected to laboratory light. The effect of chloroform in the extraction solvent was studied to ascertain whether the isomerization was due to the chloroform or the presence of an impurity or reaction product in the reagent grade chloroform used.

METHOD

Reagents

(a) *Solvents.*—Methyl *t*-butyl ether (MTBE), distilled in glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442). HPLC grade hexane and CH_2Cl_2 (Fisher Scientific Co., Pittsburgh, PA 15219). Reagent and Nanograde $CHCl_3$ (Mallinckrodt, Inc., St. Louis, MO 63147). Use reagent grade for other solvents.

(b) *HPLC elution solvents.*—Filter hexane and MTBE separately under vacuum through 0.45 μm Alpha 450 membrane (Gelman Instrument Co., Ann Arbor, MI 48105). Use 0.1% MTBE in hexane for analytical separations and 0.5% MTBE in hexane for semi-preparative separations of retinyl palmitate isomers.

(c) *Molecular sieve.*—Davison molecular sieve, Type 13X, 8–12 mesh (Fisher Scientific Co.) activated at 250°C overnight.

(d) *Preparation of retinyl palmitate isomer standard solutions.*—Use 9-*cis*-retinal and 13-*cis*-retinal (Hoffmann-La Roche, Inc., Nutley, NJ 07110) without further purification. Reduce with $NaBH_4$ to obtain corresponding retinol isomers. React retinol isomers with palmitoyl chloride in CH_2Cl_2 (10) to produce *cis*-retinyl palmitate isomers. Remove excess reagents and undesirable reaction products by preparative silica thin layer chromatography on poured plates (2 mm, Silica 60, PF 254, E. Merck, Darmstadt, GFR). Develop plates with cyclohexane–toluene–ethyl acetate (5 + 3 + 2) (18). Purify 9-*cis*- and 13-*cis*-retinyl palmitate prepared as described above and all-*trans*-retinyl palmitate in corn oil (1 000 000 USP units/g oil) (Hoffmann-La Roche, Inc.) by semi-preparative HPLC with a flow rate of 2.0 mL mobile phase/min. Evaluate purity of standards under analytical HPLC conditions. Store purified standard solutions at −20°C under nitrogen in hexane.

(e) *Stock retinyl palmitate solution.*—Use solution of purified all-*trans*-retinyl palmitate in hexane (41 μg/mL) as standard for comparison with other solvents throughout study.

Apparatus

Equipment specified is not restrictive; other suitable equipment may be used.

(a) *Analytical liquid chromatograph.*—Model 312 (Altex Scientific, Inc., Berkeley, CA 94710), with Model 110A pump and Model 210 sample injection valve (20 μL loop). Fluoro-Monitor fluorescence detector (American Instrument Co., Silver Spring, MD 20910). Corning 7-51 excitation filter (365 nm max.), Wratten 8 emission filter (500 nm, sharp cut), 70 μL flow cell, G.E. F4T4-BL lamp (American Instrument Co.).

(b) *Semi-preparative liquid chromatograph.*—Apparatus as described above with 2000 μL sample loop and Altex Model 153 analytical ultraviolet detector (8 μL flow cell, 254 nm filter).

(c) *Analytical HPLC column.*—Supelcosil LC-Si (5 μm, 4.6 mm × 150 mm) analytical HPLC column. Guard column (4.6 mm × 50 mm) tap-filled with Pelliguard LC-Si (40 μm) packing (Supelco, Inc., Bellefonte, PA 16823).

(d) *Semi-preparative column.*—LiChrosorb Si-60-5 (9 mm × 250 mm) column (Chrompack U.S.A., Inc., Whittier, CA 90607).

Procedure

(a) *Purification and stabilization of $CHCl_3$.*—Redistill reagent grade $CHCl_3$ (bp 59–62°C). Add 0.5% absolute ethanol or 50 ppm amylene (2-methyl-2-butene). Store over Type 13X molecular sieve (15).

(b) *Effect of solvent on isomerization.*—Dilute 5 mL stock retinyl palmitate solution in 3 separate 25 mL volumetric flasks and dilute to volume with hexane, stabilized $CHCl_3$, and CH_2Cl_2. Subject solutions to gold fluorescent laboratory light (Sylvania, F40G0, GTE Products Corp., Danvers, MA 01923). Distance from solutions to light source is 1.7 m. Take 5 mL aliquots at 2 h, 4 h, and 6.5 h. Evaporate solvent under nitrogen at 35°C. Redissolve treated samples in hexane and inject 20 μL (162 ng, 0.312 nmoles) into HPLC system. Use flow rate of 1.2 mL mobile phase/min.

(c) *Effect of light on isomerization.*—Follow protocol described above except sample duplicate aliquots. Subject one set of samples to gold fluorescent laboratory light for fixed time of 3.5 h and store duplicate samples in dark for same time period. Use hexane, CH_2Cl_2, MTBE, and purified, stabilized $CHCl_3$ as dilution solvents.

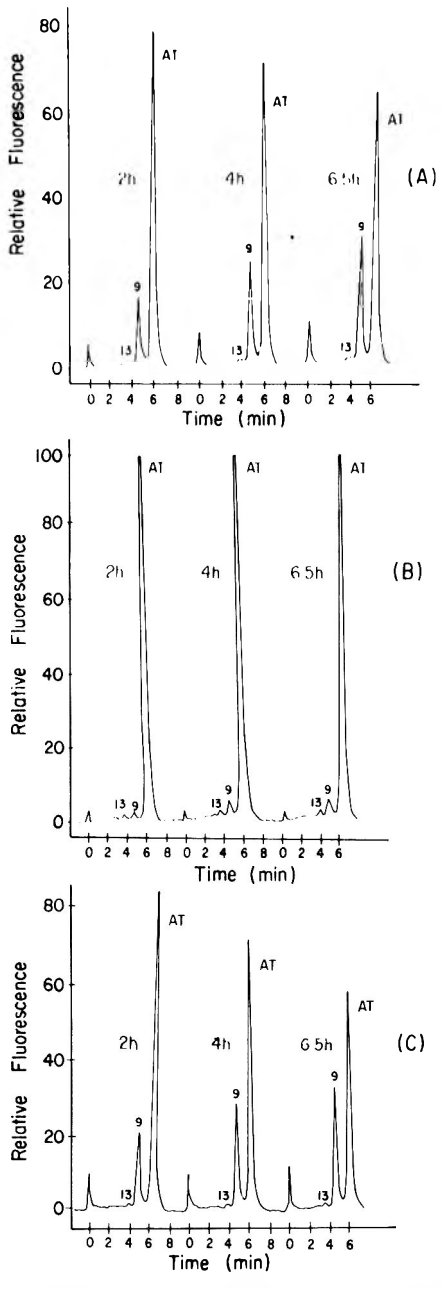

Figure 1. Representative chromatograms of retinyl palmitate in CH_2Cl_2 (A), hexane (B), and $CHCl_3$ (C) subjected to gold fluorescent laboratory light for 2 h, 4 h, and 6.5 h. 9 = 9-*cis*-retinyl palmitate; 13 = 13-*cis*-retinyl palmitate; AT = all-*trans*-retinyl palmitate.

Results and Discussion

Isomerization of retinyl palmitate extracts using chloroform in the extraction solvent system was shown to occur. It was not known whether the isomerization was due to an impurity, a degradation product in the chloroform, or some other factor.

The first experiment was performed to test various solvent purification procedures such as addition of ethanol or amylene and storage over molecular sieve to remove degradation products. Methylene chloride was tested to determine its effect on isomerization because it has been demonstrated to be a less toxic solvent than chloroform and thus is preferred for conventional lipid extractions (19).

The standard retinyl palmitate solutions used in this study were subjected to gold fluorescent laboratory light to simulate exposure to light encountered during routine extraction and analysis of vitamin A. Sylvania gold fluorescent tubes are routinely used in most laboratories where vitamin analyses are performed. Light of less than 500 nm wavelength is excluded because it may cause degradation and/or isomerization of vitamins.

The chromatograms in Figure 1 indicate that exposure of all-*trans*-retinyl palmitate to gold fluorescent laboratory light for as little as 2 h produced a pronounced change in the isomeric nature of the retinyl palmitate extracted in methylene chloride or chloroform. Isomerization was minimal when hexane or MTBE was used as solvent.

There was a noticeable increase in the 9-*cis*-retinyl palmitate peak and a corresponding decrease in the all-*trans*-retinyl palmitate concentration with increased storage time in both chloroform and methylene chloride, compared with hexane. The isomerization process occurs only when the retinyl palmitate is present in the chlorinated solvents. If these solvents are stripped under nitrogen and the retinyl palmitate is redissolved in hexane, then no further detectable isomerization is observed. Impurities or degradation products in the chlorinated solvents (e.g., HCl) do not appear to be the cause of retinyl palmitate isomerization, because changing the grade or purifying the solvent as described in the procedure had no effect on the isomerization reaction.

Identification of the peaks in the chromatograms as isomers of retinyl palmitate was made by matching the appropriate retention time with those of a standard mixture of the 3 purified isomers under the HPLC conditions. Fractions corresponding to the individual isomers were isolated under the semi-preparative HPLC conditions. The absorption maxima of the fractions

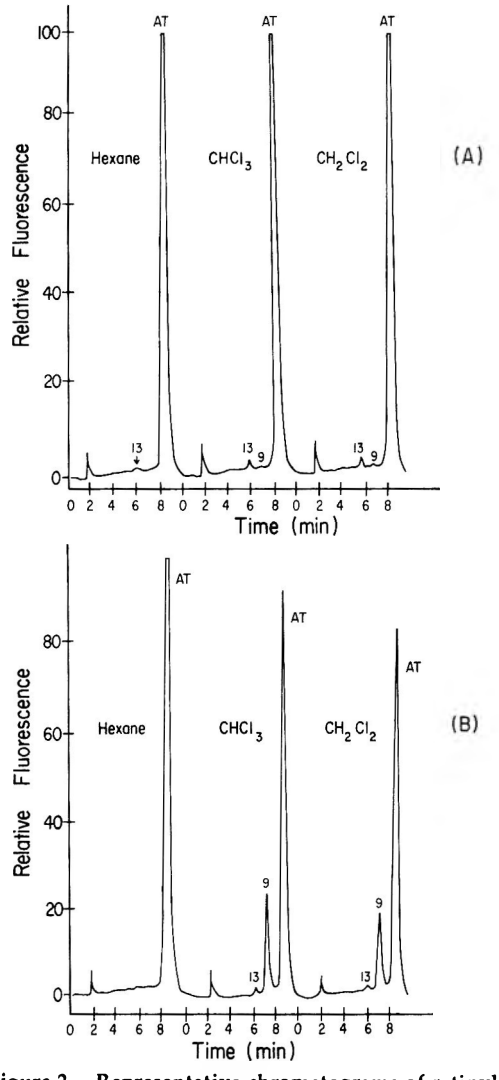

Figure 2. Representative chromatograms of retinyl palmitate in hexane, $CHCl_3$, or CH_2Cl_2 kept in absence of light (A) or in presence of gold fluorescent laboratory light (B) for 3.5 h. Other conditions same as Figure 1.

corresponded to the appropriate isomer as tentatively identified: 13-*cis*, 328 nm; 9-*cis*, 322 nm; all-*trans*, 326 nm (18). Treatment of each isolated fraction with dilute iodine produced an equilibrium mixture of the 3 isomers plus a fourth peak, possibly the 9-*cis*,13-*cis*-retinyl palmitate (8).

The second study was done to determine if the isomerization was a result of an interaction between solvent and light exposure. The results from this study (Figure 2) suggest that isomerization of retinyl palmitate is due to an interaction between light exposure and the chlorinated solvents. The presence of light had little effect on the isomerization in hexane. The results for MTBE (not shown in Figure 2) as a solvent were the same as for hexane. Likewise, chlorinated solvents stored in the dark did not promote isomerization of the retinyl palmitate during sample preparation.

Isomerization appears to be due to a photochemically induced free-radical reaction in the chlorinated solvents. Thus, if chlorinated solvents are used, all extractions should be performed in the absence of light, which would be prohibitive for the routine extraction of food products. A more acceptable alternative is the use of solvent mixtures such as hexane–MTBE or hexane–isopropanol (20) which do not affect the isomerization of vitamin A esters.

Conceivably, this phenomenon could occur with other conjugated lipid compounds such as carotenoids and unsaturated fatty acids. A preliminary investigation in our laboratory demonstrated no effect of chloroform or methylene chloride on the isomerization of methyl linoleate or linoleic acid, using capillary gas chromatographic analysis following extraction. This does not eliminate the possibility of isomerization of lipid extracts (formation of *trans*-isomers) containing highly unsaturated fatty acids when chlorinated solvents are used in conventional lipid extractions.

In conclusion, it has been demonstrated that retinyl palmitate, a conjugated lipid compound, is susceptible to photochemically induced isomerization when chlorinated solvents are used (under the conditions described simulating lipid extraction under gold fluorescent lights). Other solvents such as MTBE and hexane were not shown to be promoters of isomerization under gold fluorescent laboratory light.

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Analysis of Fat-Soluble Vitamins. XXVIII. High Performance Liquid Chromatographic Determination of Vitamin D in Pet Foods and Feeds: Collaborative Study

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A high performance liquid chromatographic (HPLC) method for vitamin D in pet foods and feeds at low concentrations (2–8 IU/g = 50–200 ppb) was studied collaboratively. The procedure consists of the following purification steps: saponification, extraction of the unsaponifiable fraction, chromatography on alumina, cleanup on reverse phase HPLC, and quantitation with straight phase HPLC. The original method, developed by Knapstein, was simplified by deleting the quantitative TLC step. Six coded samples were distributed to 31 laboratories, along with a known sample containing 15 IU/g to allow practice of the rather complicated procedure. Eighteen collaborators returned their results. Results for the spiked samples show good recovery. The estimates of repeatability and reproducibility are 0.96 and 2.2 IU/g for spiked samples and 1.5 and 3.1 IU/g for commercial samples, respectively, which are considered acceptable for these low concentrations. The method has been adopted official first action.

The high performance liquid chromatographic (HPLC) method for determining vitamin D[1] in pet foods and feeds was presented in 1980 by the Associate Referee during the 94th Annual Meeting of AOAC and recommended for collaborative study. The method is based on one developed by Knapstein and co-workers (1) from the Agricultural Research Station in Kiel, GFR, for the determination of vitamin D in feeds, which used the AOAC **43.A02(b)** and **(c)** (2, 3) cleanup and analytical columns, respectively. Extra cleanup steps consisting of alumina column chromatography and quantitative thin layer chromatography (TLC) completed the method. The quantitative TLC step, in particular, requires specially trained personnel; on request of the majority of interested collaborators, this step was omitted after evaluation by the Associate Referee.

A comparison with the AOAC biological method (4) for vitamin D was considered superfluous, since the HPLC method was tested on synthetic mixtures with a known amount of vitamin D_3, so the systematic error could be calculated.

Collaborative Study

Samples

In this study, 4 preparations and a practice sample of different composition (Table 1) were assayed. Three samples contained pure cholecalciferol in coated form (microbeadlets). Samples 1 and 2 and the practice sample were based on a layer feed (German type). The vitamin D_3 content of these samples was calculated on a weight basis. Single determinations were carried out in the coded samples. Sample 3 was based on broiler feed (German type), Sample 4 was based on cat food (Dutch type). Preparations 3 and 4 (both containing vitamin D_3 resin) were commercially available products from different manufacturers. From each of the Preparations 3 and 4, two differently coded samples were assayed, thus yielding independent duplicate determinations for each of these preparations.

The practice sample, non-coded and with given potency and chromatograms, was used to familiarize the analyst with the delicate procedure and to give "instant" confidence in the result.

For part XXVII, see de Vries, E. J., & Borsje, B. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1228–1234.

[1] Vitamin D is used here as a generic term for vitamin D_2 (ergocalciferol) or D_3 (cholecalciferol); pre-vitamin D is used for pre-vitamin D_2 or D_3.

This report of the Associate Referee, E. J. de Vries, was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

Received September 1, 1981.

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.* (1982) **65**, 338, 378.

Table 1. Composition of collaborative samples

Sample	Vit. D_3, IU/g	Preparation
Practice	exactly 15.0	layer feed[a]
1	exactly 4.0	layer feed[a]
2	exactly 7.0	layer feed[a]
3	about 3.0	broiler feed[a]
4	about 2.0	cat food[b]

[a] Containing also vitamins A, E, K_3, B_1, B_2, B_6, B_{12}, pantothenic acid, folic acid, choline chloride, and canthaxanthin.

[b] Containing also vitamins A, E, K_3, B_1, B_2, B_{12}, niacin, and choline chloride.

Collaborators

For this collaborative study, we distributed 217 samples to 31 collaborators. The collaborators were asked to test their equipment for separation of *trans*-vitamin D_3/pre-vitamin D_3 on the analytical column. If the peak resolution exceeded 1, the column performance was satisfactory.

Vitamin D in Mixed Feeds, Premixes, and Pet Foods

Liquid Chromatographic Method

Official First Action

(Applicable to products contg <200 IU and >2 IU vitamin D/g. For products contg ≥200 IU vitamin D/g, use **43.A01-43.A08.**)

43.C01 ***Principle***

Samples are saponified and extd, and unsaponifiable material is chromatographed successively on alumina to remove tocopherols and carotenes, if present, and on LC cleanup column to sep. from interfering substances. Second LC column packed with silica seps vitamin D from impurities. Vitamin D is corrected for amt previtamin D formed during saponification. Vitamin D is sum of vitamin D and previtamin D.

43.C02 ***Reagents***

(**a**) *Solvents.*—MeOH, alcohol, CH_3CN, toluene, peroxide- and acid-free ether, *n*-hexane (spectroquality). Dry *n*-hexane by passing thru column 60 × 8 cm diam. contg 500 g 50–250 μm silica dried 4 h at 150°.

(**b**) *Sodium ascorbate soln.*—Dissolve 3.5 g ascorbic acid in 20 mL 1N NaOH. Prep. fresh daily.

(**c**) *Antioxidant solution.*—1 mg butylated hydroxytoluene (BHT)/mL hexane.

(**d**) *Petroleum ether.*—Reflux over KOH pellets and collect fraction distg between 40° and 60°.

(**e**) *Ether-petroleum ether eluants.*—8 + 92 and 40 + 60.

(**f**) *Alumina.*—Neut., type 1097 (E. Merck, Darmstadt, GFR).

(**g**) *Mobile phase (for cleanup column).*—CH_3CN-MeOH-H_2O (50 + 50 + 5).

(**h**) *Mobile phase (for analytical column).*—*n*-Hexane contg 0.35% (v/v) *n*-amyl alcohol.

(**i**) *Vitamin D std solns.*—USP Ref. Std Ergocalciferol (if sample labeled as contg vitamin D_2) or Cholecalciferol (if labeled as contg vitamin D or D_3). Accurately weigh ca 12.5 mg vitamin D std (*W'*) in 100 mL amber vol. flask. Dissolve without heat in toluene and dil. to vol. with toluene (125 μg/mL, soln A). Dil. 10 mL soln A to 100 mL with mobile phase (**h**). Dil. 10 mL of this soln to 100 mL with toluene–mobile phase (**h**) (5 + 95) for vitamin D std (soln B) (1.25 μg/mL; 50 IU/mL). Also dil. 10 mL soln A to 100 mL with mobile phase (**g**); dil. 20 mL of this soln to 100 mL with mobile phase (**g**) (2.5 μg/mL, soln C). Prep. fresh daily.

(**j**) *System suitability std soln.*—Use USP Vitamin D Assay System Suitability Ref. Std, or prep. soln contg 2 mg vitamin D_3 and 0.2 mg trans-vitamin D_3/g in vegetable oil. Peaks of trans-vitamin D_3 and previtamin D_3 must have ca same peak hts. If necessary, increase previtamin D_3 content by warming oil soln ca 45 min at 90°. Store soln at 5°.

43.C03 ***Alumina Column***

Seal coarse fritted glass disk in lower end of 150 × 20 mm (id) tube and 250 mL bulb at upper end. Fit constricted portion at lower end with Teflon stopcock.

Heat 250 g alumina overnight at 750°. Cool and store in vac. desiccator. Weigh 30 g dried alumina into 100 mL erlenmeyer. Pipet 2.7 mL H_2O into flask, and stopper. Heat 5 min on steam bath. Vigorously shake warm flask until powder is free-flowing. Cool and let stand 30 min.

Add 40 mL pet ether to deactivated alumina, swirl, and transfer to tube, using pet ether. Let packing settle. Maintain head of >0.5 cm liq. on column thruout assay (alumina column can be used for only 1 assay).

43.C04 ***Liquid Chromatography***

(**a**) *Liquid chromatograph.*—Hewlett-Packard 1010A, or equiv., with 254 nm UV detector with 2 columns: cleanup and analytical.

(b) *Cleanup column.*—Stainless steel, 250 × 4.6 (id) mm, packed with 10 μm particle size Li-Chrosorb RP-18. Typical operating conditions: chart speed, 1 cm/min; eluant flow rate, 1.4 mL/min; detector sensitivity, 0.08 AUFS; temp., ambient; valve injection vol., 500 μL; solv. system, CH_3CN-MeOH-H_2O (50 + 50 + 5).

(c) *Analytical column.*—Stainless steel, 250 × 4.6 (id) mm, packed with 5 μm particle size Partisil-5, passing system suitability test. Typical operating conditions: chart speed, 1 cm/min; eluant flow rate, 2.6 mL/min (ca 1500 psi); detector sensitivity, 0.008 AUFS; temp., ambient; valve injection vol., 200 μL; solv. system, *n*-hexane contg 0.35% (v/v) *n*-amyl alcohol.

43.C05 *System Suitability Test for Analytical Column*

Dissolve 0.1 g system suitability std soln in 100 mL toluene–mobile phase (5 + 95) and inject 200 μL. Det. peak resolution between previtamin D_3 and trans-vitamin D_3 as: $R = 2D/(B + C)$; where D = distance between peak max. of previtamin D_3 and trans-vitamin D_3; B = peak width of previtamin D_3; and C = peak width of trans-vitamin D_3. Performance is satisfactory if R is ≥ 1.0.

43.C06 *Calibration*

Inject 500 μL vitamin D std soln (soln C) onto cleanup column thru sampling valve and 200 μL soln B onto analytical column, and adjust operating conditions of detector to give largest possible on-scale peaks of vitamin D. Det. retention time of vitamin D on cleanup and analytical columns and peak ht of vitamin D on analytical column. Retention time of vitamin D on cleanup column should be between 15 and 25 min; adjust H_2O content of mobile phase, if necessary, to achieve this situation. Retention time of vitamin D on analytical column should be between 15 and 20 min; adjust amyl alcohol content in mobile phase, if necessary, to achieve this situation.

43.C07 *Preparation of Sample*

Isolation of unsaponifiable matter from powder.—Accurately weigh ca 25 g powdered sample (preferably particle size <1 mm) into saponification flask. Add 80 mL alcohol, 2 mL Na ascorbate soln, a pinch of Na_2EDTA, and 10 mL 50% aq. KOH soln. Reflux 30 min on steam bath under N with mag. stirring. Cool and ext with five 60 mL portions of ether in saponification flask; decant each time and transfer ether layer to 1 L separator contg 100 mL H_2O. Shake ether layer in separator (A), let sep., and transfer aq. phase to 500 mL separator (B). Ext aq. phase with 60 mL ether and transfer ether layer to separator (A). Wash combined ether exts with 100 mL 0.5N KOH soln and then 100 mL portions of H_2O until last washing is neut. to phthln. Add 150 mL pet ether, wait ½ h, sep. from last drops of H_2O, and add 2 sheets of 9 cm filter paper in strips to separator. Shake, add 1 mg BHT, and transfer to r-b flask, rinsing separator and paper with pet ether.

Evap. soln by swirling (Rotavapor) under N stream in 40° H_2O bath. Dissolve residue immediately in 5 mL hexane.

43.C08 *Alumina Column Chromatography*

Transfer sample soln to column with aid of three 10 mL portions of hexane. Discard eluate (contains carotenoids). Elute column with seven 10 mL portions of ether–hexane (8 + 92) and discard eluate (contains tocopherols and ethoxyquin).

Elute column with seven 10 mL portions of ether–hexane (40 + 60), discard first 20–25 mL, collecting rest of eluate in r-b flask (contains vitamins A and D) when front of fluorescent vitamin A band is located 3 cm from bottom of column. Examine column <1 s under UV light (360 nm) with portable UV lamp to verify elution of vitamin A. Evap. soln by swirling (Rotavapor) under N stream in 40° H_2O bath. Transfer to centrf. tube, rinsing flask with 2–3 mL ether, evap. ether, and dissolve in 1.0 mL MeOH with warming. Add 1.0 mL CH_3CN and cool. Centrf. and use clear supernate for injection onto cleanup column.

43.C09 *Determination*

(a) *Cleanup.*—Inject 500 μL sample soln onto cleanup column thru sampling valve and adjust operating conditions of detector to give largest possible on-scale peaks for vitamin D. Collect fraction between 3 min before and 3 min after vitamin D peak in 10 mL vol. flask. Add 1 mL antioxidant soln and evap. to dryness under N stream. Dissolve residue immediately in 2.0 mL toluene–mobile phase (5 + 95). Use this soln for injection onto analytical column.

(b) *Assay.*—Inject 200 μL soln (a) onto analytical column thru sampling valve, and adjust operating conditions of detector to give largest possible on-scale peaks of vitamin D. Measure peak ht of vitamin D. Use same operating conditions and inject std soln B. Measure peak ht of

Table 2. Columns and packings used for cleanup column

Lab.	Column, cm × mm	Packing	Particle size, μm	Mobile phase CH_3CN–MeOH–H_2O	Flow rate, mL/min	Ret. time vit. D, min
1	25 × 4.6	R-Sil C18 HL	10	50 + 50 + 5	1.4	23
2	25 × 3.0	LiChrosorb RP18	10	50 + 50 + 5.5	0.9	17
3	25 × 4.0	LiChrosorb RP18	5	50 + 50 + 6	1.4	18
4	25 × 4.6	LiChrosorb RP18	10	50 + 50 + 4	1.4	17
5	30 × 4.0	Micropak MCH10	10	50 + 50 + 6	1.5	15
6	30 × 3.9	μBondapak C18	10	50 + 50 + 5	1.4	9
7	25 × 4.0	LiChrosorb RP18	10	50 + 50 + 5	1.5	14
8	25 × 4.0	LiChrosorb RP18	10	50 + 50 + 5	1.5	15
9	30 × 4.6	μBondapak C18	10	50 + 50 + 5	1.4	12
9A	30 × 4.6	μBondapak C18	10	50 + 50 + 5	1.3	12
10	25 × 4.0	LiChrosorb RP18	10	50 + 50 + 5	1.2	17
11	25 × 4.6	LiChrosorb RP18	7	50 + 50 + 2	1.0	12–15
12	30 × 3.9	μBondapak C18	10	50 + 50 + 10	1.4	13–21
13	30 × 3.9	μBondapak C18	10	50 + 50 + 5	1.4	14–19
14	10 × 8	C18 cartridge A	6	50 + 50 + 5	2.4	20
15	25 × 4.6	LiChrosorb RP18	10	50 + 50 + 5	1.8	17–19
16	25 × 4.6	Spherisorb ODS	5	100 + 0 + 0	1.2	16
17	22 × 4	LiChrosorb RP18	10	50 + 50 + 5	2	19
18	25 × 4.6	LiChrosorb RP18	10	50 + 50 + 5	1.4	20

vitamin D.

(c) *Calculation.*—

Vitamin D potency in IU/g sample

$$= \frac{1.25 \times P \times W' \times V \times 40\,000}{P' \times W \times V'}$$

where P = peak ht of vitamin D in sample soln; 1.25 = correction factor for previtamin D formed during refluxing for saponification; P' = peak ht of vitamin D in ref. soln; W = g sample weighed; W' = mg ref. std; V = total mL sample soln; V' = total mL ref. std soln; 40 000 = IU vitamin D/mg USP Ref. Std.

Results and Discussion

Collaborators' Comments

Tables 2 and 3 summarize the various columns and packings used, and the retention times and resolutions obtained in the collaborative study. Several collaborators had difficulties obtaining a water-free unsaponifiable residue and one recommended drying the extract before evaporation by filter paper, similar to AOAC **43.A07**. Some collaborators encountered difficulties dissolving the eluate from the alumina column, which they solved by either dissolving at ele-

Table 3. Columns and packings used, and resolution obtained with analytical column

Lab.	Column, cm × mm	Packing	Particle size, μm	% Amyl-alcohol	Flow rate, mL/min	Ret. time vit. D, min	Resolution pre/trans
1	25 × 4.0	LiChrosorb SI-60	5	0.3	2.6	12–13	1.0
2	25 × 4.6	R-Sil	5	0.5	2.6	18	1.23
3	25 × 4.0	LiChrosorb SI-60	5	0.35	2.5	18	1.58
4	25 × 4.6	Partisil	5	0.5	2.4	16–17	1.8
5	25 × 4.6	LiChrosorb SI-100	5	0.6	1.6	16	1.17
6	25 × 4.0	LiChrosorb SI-60	5	0.3	2.0	29	1.33
7	25 × 4.6	Spherisorb S5W	5	0.35	2.6	14–15	1.06
8	30 × 4.0	LiChrosorb SI-60	5	0.35	2.0	21	1.3
9	25 × 4.6	Partisil	5	0.7	2.5	10	—
9A	20 × 4.6	Partisil	5	1.0[a]	2.0	6	—
10	25 × 4.0	LiChrosorb SI-60	5	0.35	2.4	19	1.65
11	20 × 4.6	Partisil	5	0.35	2.5	14	1.7
12	30 × 3.9	μPorasil	10	0.35	2.5	18	1.2
13	30 × 3.9	μPorasil	10	0.35	2.6	19	1.0
14	25 × 4.6	Zorbax-silica	6	0.4	3.0	20	1.3
15	25 × 4.6	Partisil	5	0.35	2.4	19–22	—
16	25 × 4.6	LiChrosorb SI-60	10	0.4	3.0	18–21	1.0
17	22 × 4	Partisil	5	1.0	2.0	13	—
18	25 × 4.6	Partisil	5	0.5	2.0	22	—

[a] Ethanol in hexane.

Table 4. Collaborative results (IU/g) for determination of vitamin D_3 in foods and feeds

	Samples						
Lab.	1	2	3		4		Practice
1	—[a]	5.3	13.6	—[a]	2.8	5.7	17.9
2	4.4	6.7	3.3	2.1	4.9	2.4	14.2
3	4.0	6.4	11.1	7.8	2.6	7.6	14.7
4	4.0	6.3	5.2	1.9	0.9	0.5	14.6
5	8.7	13.3	5.7	6.2	7.2	5.4	21
6	3.7	6.9	3.2	2.7	1.1	1.2	14.8
7	2.9	7.5	3.6	6.0	2.6	5.4	22.9
8	6.4	8.8	2.9	3.2	2.8	2.7	—
9	3.8	6.9	3.6	2.7	—[b]	—[b]	15.3
9A[c]	4.6	7.0	3.2	3.0	1.5	0.9	16.8
10	12.8[c]	17.5[c]	10.7	13.6	2.1	2.0	16.9
11	3.6	7.1	2.7	2.5	2.2	1.6	13.5
12	8.0	9.8	5.5	9.2	3.8	1.8	11.7
13	13.2[c]	2.9[c]	3.7	2.3	7.6	3.4	19.0
14[c]	—	0	0.4	0	0.6	0.2	15.7
15	2.8	6.2	1.5	1.4	1.0	1.0	7.5
16	2.6	3.1	0.9	4.4	1.3	1.5	16.9
17	3.0	4.6	6.2[c]	15.4[c]	146.0[c]	2.0[c]	27
18	4.4	10.1	3.1	2.1	1.6	3.4	13.8
Mean	4.45	7.27	4.79		3.00		16.36

[a] D_3 peak is slight deviation on back of other peak.
[b] Failed.
[c] Excluded from statistical analysis, see text.

vated temperatures or filtering over Millipore filters.

Two collaborators stated that the method is time-consuming. One collaborator remarked that the alumina column chromatography was rather laborious, whereas another collaborator commented that this column was speeded up by using slight nitrogen pressure.

One collaborator observed some separation problems. Two collaborators questioned the necessity of measuring the pre-vitamin D. Two collaborators reported a slight difference between the retention times for vitamin D in the reference and sample solutions.

Results

The collaborative results are given in Table 4. Eighteen collaborators participated in this study. Laboratories 1 and 9 reported an incomplete set of results. Laboratory 9, which failed to complete Sample 4, also analyzed the samples according to the Knapstein method (1) and reported the results obtained under 9A; these results were excluded from the statistical evaluation. Laboratory 14 failed to recover vitamin D_3 for all samples. Those results were also excluded from statistical analysis.

Statistical Evaluation

The collaborative study included the analysis of 4 preparations of different composition (see Table 1). Preparations 1 and 2 were identical except for the vitamin D content (4.0 and 7.0 IU/g, respectively). From each of the preparations 3 and 4, 2 differently coded samples were sent to collaborators, thus yielding independent duplicate determinations.

Laboratory 1 reported that the vitamin D_3 peak was a slight deviation on the back of another peak for both Sample 1 and one of the duplicate Samples 3. Laboratory 9 reported "failed" on both Samples 4. Laboratory 14 reported less than satisfactory levels of vitamin D_3 on 5 of the unknown samples. This may be due to the unsuitable column used by this laboratory (10 cm × 8 mm). Sample 1 was consumed in testing before any quantitative result could be obtained. The poor recovery, in our opinion, does not warrant inclusion in the statistical analysis. Moreover, these results would be rejected by the ranking test for outlying laboratories. As a consequence, 4 values are missing in that part of Table 4 that is subjected to statistical analysis.

For a simple statistical analysis, equal numbers of replicate determinations per sample must be available. In this respect, single determinations of 2 samples differing in vitamin D content only may be regarded as replicates.

To detect laboratories which show consistently high or low values, the ranking test described by

Youden (5) was applied to sums of paired observations (duplicates of Samples 3 and 4, and Samples 1 and 2). The results of Laboratories 1 and 9 were excluded because of missing values.

No outlying laboratories were found (at the 5% level of significance). It is worth noting that the omission of data from Laboratories 1 and 9 would not have influenced the outcome of the ranking test, because the available results of both laboratories are, in general, not extreme.

Next, the data were examined for incidental outlying results per sample by means of Dixon's test on sums of paired observations. The test attained significance at the 5% level in 2 instances: Laboratory 10 on Samples 1 and 2 and Laboratory 17 on Sample 4. We decided to omit these extremely high results from further statistical analysis.

One of the assumptions underlying the final statistical analysis (analysis of variance) is homogeneity of variation between and within laboratories. It was expected that the spiked samples (Samples 1 and 2), having a more homogeneous distribution of vitamin D, would be assayed with better precision than the 2 commercial samples. Therefore, we decided to perform separate analyses of variance for the spiked samples and for the commercial samples.

To check the assumptions of homogeneity of variation for Samples 3 and 4, the test described by Steiner (5) was performed. This test revealed heterogeneity at the 5% level of significance on the large difference between the duplicates of Sample 3 for Laboratory 17. Both results were excluded from further statistical analysis, and the remaining results could be regarded as homogeneous according to this test.

With respect to Samples 1 and 2, the homogeneity of within-laboratory error was investigated in a similar way. As the Samples 1 and 2 can be regarded as replicates, except for the systematic difference (3 IU/g), the ranges, on which the test is based, were obtained by subtracting 3 IU/g from the differences between the results on Sample 2 and Sample 1. The test attained significance at the 5% level on the abnormal large difference for Laboratory 13. All other laboratories found a higher vitamin D_3 content on Sample 2 than on Sample 1, in sharp contrast with the strongly negative difference (Sample 2 − Sample 1) for Laboratory 13. We decided to exclude both results from Laboratory 13. The remaining residual variances could be regarded as homogeneous according to the test.

Table 5. Analysis of variance of collaborative results (IU/g) for HPLC determination of vitamin D in pet foods and feeds

Source of variation	Degrees of freedom	Mean square
Spiked Samples (Samples 1 and 2)		
Samples	1	65.59
Laboratories	14	8.88[a]
Within-laboratory error	13	0.93
Estimate of repeatability SD		s_o = 0.96 IU/g (df = 13)
Estimate of reproducibility SD		s_x = 2.2 IU/g (df[b] = 16)
Commercial Samples (Samples 3 and 4)		
Samples (S)	1	76.51
Laboratories (L)	15	21.39
L × S interaction	14	12.78[a]
Replicates	30	2.40
Estimate of repeatability SD		s_o = 1.5 IU/g (df = 30)
Estimate of reproducibility SD		s_x = 3.1 IU/g (df[b] = 35)

[a] Significant at the 1% level.
[b] Approximated by Satterthwaite's formula (6).

Table 5 shows the analyses of variance. The missing values were dealt with by the well known missing value technique, i.e., for Laboratory 1 by minimizing the replicates sum of squares and for Laboratory 9 (Sample 4) by minimizing the interaction sum of squares.

For the spiked samples the estimates of the repeatability and the reproducibility standard deviations (according to Steiner (5)) are s_o = 0.96 IU/g; s_x = 2.2 IU/g. Because the actual contents of Samples 1 and 2 are accurately known, an estimate of the bias of the method can be obtained as the mean of the deviations from the known contents. The bias with 95% confidence limits is estimated by: mean bias = 0.3 (−0.9 ~ 1.5) IU/g.

The analysis of variance for Samples 3 and 4 (Table 5) shows that the laboratory × sample interaction is statistically significant at the 1% level. As a consequence, the differences between samples cannot be regarded as constant for all laboratories. The estimates of the repeatability and reproducibility standard deviations for the commercial samples are s_o = 1.5 IU/g; s_x = 3.1 IU/g. As was expected, better precision is obtained with the spiked samples than with the commercial samples.

Discussion

The mean values for Samples 1 and 2 (Table 4) show that the added amounts of vitamin D_3 are

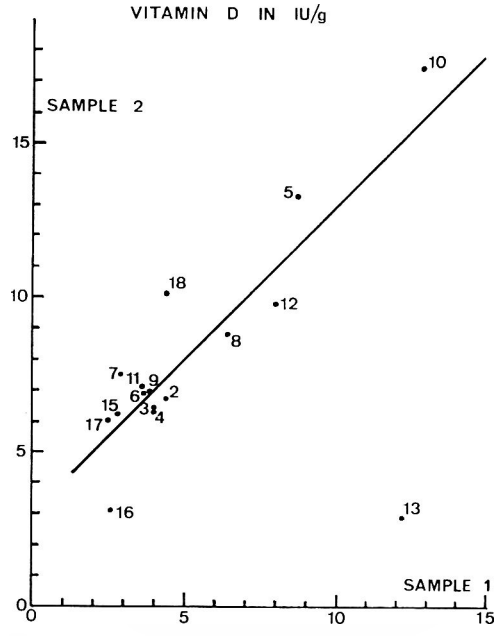

Figure 1. Two-sample plot of collaborative results on Samples 1 and 2.

recovered quite well (recoveries of 111% for Sample 1 and 104% for Sample 2), resulting in a small bias (estimated as 0.3 IU/g), which is far from statistically significant.

Sixteen laboratories reported results on both Samples 1 and 2. The 2-sample plot (Figure 1) demonstrates that 2 of the points are clearly separated from the main cluster (Laboratories 10 and 13). The remaining 14 points closely match the theoretical 45° line. This indicates that these 14 laboratories were in very good agreement on the difference in vitamin D potency between the 2 samples. In fact, the mean recovery of that difference is 99%. Therefore, we conclude that the tested method is accurate.

The difference in precision for the spiked samples and the commercial samples may be explained by the fact that Samples 1 and 2 were made on a laboratory scale, using microbeadlets, and resulting in almost homogeneous distribution of the vitamin D_3 particles over the samples, whereas Samples 3 and 4 were produced in feed plants with variable vitamin D sources and distribution patterns. Various vitamin D_3 sources have a different vitamin D_3 potency per particle; in general, vitamin D_3 beadlets are better distributed in the feed than vitamin AD_3 beadlets. For the spiked samples, a specially made vitamin D_3 beadlet (concentration about 4000 IU/g) was used.

As a result of collaborators' comments, the procedure was changed as follows: Water is removed by adding petroleum ether to the ether extract and drying with filter paper as described in **43.A07**. The alumina column should be packed in such a way that the flow rate is 3–5 mL/min; nitrogen pressure may be applied. Measurement of pre-vitamin D is superfluous, since most pre-vitamin D is removed from the vitamin D fraction in the cleanup step and the peak height is not used in the calculation.

Recommendations

It is recommended that the HPLC method for the determination of vitamin D in pet foods and feeds be adopted official first action, and that study be continued on procedures for vitamin D to simplify methods and to lower the limit of detection.

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MEAT AND MEAT PRODUCTS

Comparative Analysis of Meat Samples Prepared with Food Chopper and Bowl Cutter

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Analyses of meat samples after preparation with either a bowl cutter or by the official procedure with a food chopper were compared for homogeneity of comminution and for differences in fat, moisture, and protein content. Cutting time in the bowl cutter was limited to minimize temperature rise in samples. Beef chuck, pork shoulder, and beef shank, cheek, and tongue were used in the study. Variances of replicate analysis data for the 5 meat types were pooled for either cutter or chopper treatment and for each analyzed component. Sample portions cut and mixed by using the bowl cutter were more homogeneous than those ground with a food chopper. Comparative accuracy was indicated by fat and moisture means: 5 were in good agreement and 5 differed significantly; 3 of 5 paired protein means differed significantly but were within 0.3% protein. Results on precision and accuracy as well as the simplicity and convenience of the bowl cutter procedure favor its use as an alternative to a food chopper for preparing meat samples for analysis.

Preparation of meat samples for analysis by AOAC procedure **24.001** (1) involves passing the sample rapidly through a food chopper 3 times, using a plate with openings of $\leq 1/8$ in., with thorough mixing after each grinding. The same procedure is specified in the laboratory manual of the American Meat Institute (2) except that a plate with approximately $1/8$ in. holes is specified. The USDA laboratory manual (3) for regulatory analysis directs use of the AOAC procedure except that a plate with $5/64$ in. holes is specified. Ockerman (4), in an Ohio State University laboratory manual, omits specification of plate hole size in a procedure similar to that of AOAC, but recommends regrinding 5 times for any product that mixes poorly. This reference demonstrates the importance of regrinding in the variation of apparent fat content in a beef lot after each of 3 grinding and mixing operations, and allows 2 alternative methods of sample preparation (ground 3 times in a food chopper using a plate with $1/8$ in. holes, or reduction of meat to a very fine particle size with a bowl cutter) for determination of fat by using the modified Babcock method.

Although not currently specified as an alternative AOAC procedure, bowl cutters are commonly used for preliminary preparation of samples for analysis. Experience in this laboratory with using a bowl cutter for preparing meat emulsions indicated its utility for routine cutting and mixing of meat samples before proximate analysis. Apparent advantages are the fineness of particles and the uniformity of sample constituents. Use of a food chopper with a worn feed screw may result in sinews separating from meat tissue and wrapping around the screw or behind the cutter bar. Adipose tissue particles also may adhere to the inner surface of the barrel. The time required for preparation of a sample with either cutter or chopper is about the same (2–4 min) but the methods of action differ, with the bowl cutter knives the more efficient in dispersion and mixing.

Wijlhuizen and Paardekooper (5), using model systems, studied the parameters involved in both particle size reduction and mixing of meat in bowl cutters of different sizes. Parameters included geometry of the cutter, speed of knives and bowl, amount and initial size of meat pieces, length of time of cutting action, number of knives, and distance between knives and bowl. From their preliminary results, the investigators concluded that further study may lead to the ability to preselect conditions to achieve optimum efficiency of cutting and mixing action when the parameters are fitted in a mathematical equation. Assurance of optimum homogeneity of samples prepared for analysis would complement results reported here.

Our objective was to compare samples prepared with a bowl cutter and a food chopper, and to determine if the alternative procedure yielded a more uniform and representative product than procedure **24.001.** Both the food chopper and

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bowl cutter were benchtop models. Comparative analyses were used so that precision of proximate analysis indicated sample homogeneity within treatment procedures, and accuracy of the compared data indicated any treatment effect between procedures measured as a difference of fat, moisture, or protein content.

METHOD

Principle

Combined action of the knives and bowl cutter reduce the size and mix the particles of fat and lean tissue of a meat with high efficiency. Test portions of a cut and mixed sample may be taken for proximate analysis without manual mixing and quartering. As with other particle size reduction procedures, samples must be firmed by prechilling before cutter treatment. The sample and any cutter parts that may contact it must be precooled to −1.5 to −2.0°C to reduce melting of fatty constituents and dripping of meat juice from the shearing friction of cutter knives. The ideal sample size and length of cutting time necessary to obtain a small particle size and good mixing must be predetermined from trials for each model of bowl cutter to be used. Kilogram samples of coarse (1/2 in. plate) ground meat require 4 min treatment for beef and 2 min for pork. Over-treatment produces a glossy appearance on the sample surface indicating a temperature rise of between 0.5 and 5.0°C.

Apparatus

(a) *Bowl cutter.*—Benchtop model, 1/2 HP; 14 in. bowl, 22 rpm; two 3.5 in. knives, 1725 rpm; Model 84145, Hobart Corp., Troy, OH, or equivalent.

(b) *Food chopper.*—Benchtop model, 1/2 HP; 2.75 in. D plate with 1/8 in. D holes; Model 5126, Toledo Scale Co., Columbus, OH, or equivalent.

Preparation of Samples

Boneless beef chuck, pork shoulder, and 3 meats with high connective tissue content—beef cheek, shank, and tongue—were used in the study. Beef chuck (5.7% fat, 20.8% protein) and beef fat trimmings (78.7% fat, 3.3% protein) were coarse-ground individually through a 1/2 in. plate by using a floor model food chopper and were used to formulate 6 samples that ranged from 10 to 30% fat. Pork shoulder meat (18.0% fat, 17.5% protein) and back fat (91.3% fat, 1.8% protein) were similarly ground and used to prepare 4 samples that ranged from 20 to 50% fat. Two lots of each of the 3 high connective meats were ground in the same manner and used without added fat.

Each coarse ground sample of meat mixture or type was packaged in plastic freezer bags in 1 kg amounts for storage. Duplicate bags of each sample were flattened to a thickness of about 1/3 in. and stored at −34°C. All frozen meat samples along with contact parts of the food chopper and bowl cutter were tempered in a −1.5 to −2°C cold room overnight before cutter or chopper treatment.

Bowl cutter treatment of prechilled samples required 4 min for beef and 2 min for pork in preliminary trials. The cutter was stopped halfway through the cutting procedure to recover and to return any particles that adhered to the cover and knife-comb.

Operating time required to pass a 1 kg sample 3 times through the food chopper and to mix the ground meat between passes was approximately 4 min.

A sampling procedure for analysis of the prepared meat samples was devised to permit comparison of data within and between treatment procedures. To determine homogeneity of meat treated in the bowl cutter, one 100 g sample was taken from each quadrant for analysis because the entire quantity in the bowl was not manually mixed. Meat ground in the food chopper was sampled randomly and mixed thoroughly as is commonly done to obtain 200 g of a composite analytical sample.

Determinations

Fat, moisture, and protein content were determined by using methods **24.005(a), 24.003(a),** and **24.027,** respectively (1), on all analytical samples of formulated and of unmodified meats. Single or duplicate determinations were made on each of the bowl cutter quadrant samples and replicate determinations (2–8 for beef and 4 for the other meat samples) were made on food chopper composites.

Statistical Treatment

Data from the comparative analysis of beef chuck (Table 1), pork shoulder (Table 2), and high connective tissue meat samples (Table 3) were analyzed statistically for variations within (Table 4) and differences between (Table 5) bowl cutter and food chopper treatments. A 95% probability confidence level was selected. Variance of replicate analyses (precision) indicated homogeneity within a prepared sample.

Table 1. Proximate analysis of beef chuck samples [a]

Fat level, %	Cutter			Chopper		
	Fat	Moisture	Protein	Fat	Moisture	Protein
30	30.6	53.6	15.0	30.6	53.9	15.0
	30.2	53.7	14.9	30.2	54.0	15.1
	30.7	53.8	15.2	32.0		15.1
	30.6	53.8	15.4	30.8		15.3
		54.0				
		54.0				
		54.1				
		53.8				
26	26.8	56.6	16.3	27.0	56.5	16.1
	27.5	56.0	15.8	26.0	55.9	15.9
	27.1	56.3	15.8	27.3		16.0
	26.9	55.8	15.9	26.4		16.0
		56.8				
		56.2				
		56.3				
		56.2				
22	22.7	59.8	17.0	22.3	59.8	17.0
	22.6	59.8	16.8	22.3	60.4	17.2
	23.2	59.8	16.9	22.7	60.4	17.0
	23.0	59.9	16.7	23.0		16.9
		59.7				
		59.8				
		59.9				
		59.9				
		59.7				
		59.8				
		60.0				
		59.9				
18	18.5	63.2	17.9	18.3	63.0	18.0
	18.4	63.1	18.0	18.6	63.0	18.0
	18.9	63.0	18.1	18.4	62.8	18.1
	18.7	63.0	18.0	18.8		18.0
		62.9				
		63.2				
		63.0				
		63.0				
14	15.5	65.8	18.8	14.7	65.7	18.8
	14.8	65.8	18.8	14.0	66.0	19.0
	15.4	65.8	19.0	14.5	66.6	18.8
	15.0	65.5	18.8	13.9	66.0	18.7
		65.8		15.0	66.2	18.7
		65.7		14.3	66.3	18.9
		65.9		15.6		18.8
		65.8		14.6		18.7
10	10.8	68.7	19.8	10.7	68.8	19.9
	11.2	68.6	19.8	11.3	68.5	19.7
	10.6	69.0	20.2	10.6		19.6
	10.9	68.7	19.7	12.1		19.5
		68.7				
		68.6				
		68.8				
		68.8				

[a] Results (%) of replicate determinations.

Paired variances from chopper and cutter treatments were compared for significant difference before and after pooling data according to 3 experimental variables: treatment (cutter or chopper), analyzed component, and fat level or sample type. Analysis of variance compared means of replicate data for each sample group and each component analysis (accuracy). This comparison of means could be viewed as a strong indication of when loss of tissue components occurred (treatment effect) in either the bowl cutter or food chopper except that a difference between results is compounded by inseparable formulating and sampling errors.

Table 2. Proximate analysis of pork shoulder samples [a]

Fat level, %	Cutter			Chopper		
	Fat	Moisture	Protein	Fat	Moisture	Protein
50	48.8	39.7	10.8	50.5	39.5	11.0
	49.0	39.8	10.9	51.2	38.1	10.8
	49.5	39.6	10.7	48.8	37.6	10.7
	49.3	39.5	10.9	49.7	38.2	11.0
40	39.4	47.1	13.4	38.4	47.8	13.4
	39.2	47.5	13.2	38.6	47.1	12.9
	39.2	47.3	13.1	38.8	47.7	13.1
	39.4	47.6	13.1	38.4	47.7	13.0
30	29.6	54.8	15.0	30.2	54.9	14.8
	29.6	54.7	15.0	29.8	53.8	14.9
	29.5	54.9	15.3	29.4	54.3	15.1
	29.4	54.9	15.0	29.8	54.7	14.9
20	19.5	62.8	17.0	19.4	62.5	17.2
	19.5	62.6	17.1	19.4	62.8	17.5
	19.8	62.5	17.0	19.9	62.2	17.3
	19.4	62.6	17.2	19.9	62.7	17.1

[a] Results (%) of replicate determinations.

Results and Discussion

Variance of Replicate Analysis

Fat Determinations.—Variances of replicate determinations (Table 4) were random with only slight indication of proportionality to fat content. The 16 values ranged from 0.001 to 0.104 on cutter-treated meats and 0.041–1.120 on chopper-treated meats. Variances of replicates of chopper composite samples were higher than those of cutter quadrant samples in 15 of the 16 paired comparisons of which 7 were significantly higher. When variances were pooled across fat content for chopper and cutter paired groups (5 meat types), values for each of the 5 chopped meats were higher than the paired cutter value and 4 of the 5 were significantly higher.

Moisture Determinations.—Variances of repli-

Table 3. Proximate analysis of beef meats with high connective tissue content—shank, cheek, and tongue [a]

Sample type	Cutter			Chopper		
	Fat	Moisture	Protein	Fat	Moisture	Protein
Shank 1	3.9	74.1	21.2	4.1	74.1	21.1
	4.0	74.1	21.0	4.1	74.0	21.0
	4.0	74.1	20.9	4.4	74.4	21.1
	4.0	74.3	20.9	3.9	74.4	21.3
2	3.7	74.2	21.3	3.7	73.6	22.0
	3.8	74.2	21.1	3.8	74.1	22.1
	3.8	74.3	21.3	4.3	73.6	21.7
	3.8	74.3	21.5	4.2	73.9	21.9
Cheek 1	12.0	67.9	19.3	11.4	68.5	19.7
	12.7	67.9	19.1	12.0	68.9	20.0
	12.2	67.9	19.1	11.4	68.8	19.6
	12.2	67.8	19.4	11.2	68.7	19.9
2	14.2	66.4	18.9	13.2	66.7	18.4
	14.3	66.1	19.2	13.0	66.3	19.4
	14.4	66.3	19.1	13.0	66.6	19.3
	14.4	66.3	19.2	13.8	67.1	18.9
Tongue 1	19.0	64.2	15.9	19.9	63.7	15.8
	19.0	64.4	15.8	19.3	64.0	15.8
	18.9	64.2	16.0	19.9	64.5	15.7
	18.9	64.1	15.9	18.9	63.9	15.5
2	15.7	66.8	17.0	16.0	66.7	16.9
	15.4	66.9	17.1	16.0	67.0	17.1
	15.3	66.8	17.2	15.7	66.6	16.7
	15.5	66.6	17.0	16.6	67.1	17.0

[a] Results (%) of replicate determinations.

Table 4. Variance and homogeneity test of preparation procedure

Fat level (%) or sample type	Fat			Moisture			Protein		
	Cutter	Chopper	*F*-value	Cutter	Chopper	*F*-value	Cutter	Chopper	*F*-value
Beef Chuck									
30	0.056	0.599	10.8[a]	0.023	0.009	2.8	0.036	0.013	2.7
26	0.090	0.312	3.5	0.109	0.205	1.9	0.054	0.003	20.7[a]
22	0.079	0.112	1.4	0.008	0.127	15.9[a]	0.012	0.009	1.4
18	0.060	0.055	1.1	0.011	0.019	1.8	0.013	0.003	4.6
14	0.104	0.305	2.9	0.010	0.087	8.8[a]	0.014	0.011	1.3
10	0.059	0.488	8.2	0.014	0.061	4.3	0.048	0.027	1.8
Sum of degrees of freedom	18	22		46	12		18	22	
Pooled variance	0.075	0.311	4.2[a]	0.03	0.08	3.1[a]	0.029	0.011	2.7[a]
Pork Shoulder									
50	0.087	1.120	12.9[a]	0.015	0.627	43.3[a]	0.008	0.014	1.7
40	0.016	0.041	2.5	0.044	0.079	1.8	0.015	0.038	2.6
30	0.007	0.105	14.6[a]	0.010	0.219	22.4[a]	0.024	0.022	1.1
20	0.039	0.084	2.2	0.018	0.060	3.3	0.013	0.019	1.5
Sum of degrees of freedom	12	12		12	12		12	12	
Pooled variance	0.037	0.337	9.1[a]	0.022	0.246	11.4[a]	0.015	0.023	1.6
Beef Shank, Cheek, and Tongue									
Shank 1	0.003	0.047	15.8[a]	0.007	0.040	6.2	0.023	0.022	1.0
2	0.001	0.089	63.9[a]	0.003	0.057	19.0[a]	0.029	0.029	1.0
Sum of degrees of freedom	6	6		6	6		6	6	
Pooled variance	0.002	0.068	31.1[a]	0.005	0.049	10.1[a]	0.026	0.026	1.0
Cheek 1	0.088	0.135	1.5	0.001	0.031	38.6[a]	0.015	0.035	2.3
2	0.007	0.127	17.3[a]	0.015	0.114	7.6	0.012	0.070	6.1
Sum of degrees of freedom	6	6		6	6		6	6	
Pooled variance	0.048	0.131	2.8	0.008	0.072	9.2[a]	0.014	0.052	3.9
Tongue 1	0.001	0.241	185.7[a]	0.008	0.106	12.7[a]	0.006	0.022	3.9
2	0.025	0.128	5.1	0.015	0.051	3.3	0.011	0.029	2.6
Sum of degrees of freedom	6	6		6	6		6	6	
Pooled variance	0.013	0.185	13.9[a]	0.012	0.079	6.7[a]	0.008	0.025	3.1

[a]Exceeds tabular *F*-value at $P = 0.05$.

cate determinations (Table 4) were random with slight indication of an inverse relation to moisture level. The 16 values ranged from 0.001 to 0.109 for cutter-treated meats and 0.009–0.627 for chopper-treated meats. Variances of replicates of chopper composite samples were higher than those of cutter quadrant samples in 15 of the 16 paired comparisons of which 7 were significantly higher. When variances were pooled across moisture content for chopper and cutter paired groups, values of all 5 chopper-treated meats were significantly higher than the paired cutter value.

Protein Determinations.—Variances of replicate Kjeldahl determinations (Table 4) were random and varied independent of protein content. The 16 values ranged from 0.006 to 0.054 for cutter-treated meats and 0.003–0.070 for chopper-treated meats. Variances of replicates of chopper composite samples were higher than those of cutter quadrant samples in 8 of the 16 paired comparisons and 1 was significantly lower. When variances were pooled across protein content for chopper and cutter paired groups, the values for 3 of the 5 meats treated with chopper were higher and 1 was significantly lower than their paired cutter values. The latter low variance (chopper-treated beef chuck) contrasts with the comparisons of pooled variances of fat and moisture determinations, but may result from the generally low variances associated with the Kjeldahl method.

Analysis of Variance of Means

Analysis of variance of fat determinations (Table 5) indicated no treatment effect on the fat content of beef chuck, pork, and shank meat samples. Fat in chopper-treated portions of cheek meat, however, was determined 0.9% high and in tongue 0.6% low, both significant. The

Table 5. Means (%) of replicate determinations and tests of treatment effects by analysis of variance on paired data

Fat level (%) or sample type	Fat			Moisture			Protein		
	Cutter	Chopper	*F*-value	Cutter	Chopper	*F*-value	Cutter	Chopper	*F*-value
Beef Chuck									
30	30.5	30.9	0.8	53.8	53.9	0.5	15.1	15.1	0.1
26	27.1	26.7	1.7	56.3	56.2	0.1	16.0	16.0	0.2
22	22.9	22.6	1.9	59.8	60.2	11.5[a]	16.8	17.0	7.5[a]
18	18.6	18.5	0.6	63.0	62.9	2.5	18.0	18.0	0.7
14	15.2	14.6	4.2	65.8	66.1	10.9[a]	18.8	18.8	0.3
10	10.9	11.2	0.6	68.7	68.6	0.7	19.9	19.7	2.5
Overall mean	20.9	20.7	1.6	61.2	61.3	5.9	17.4	17.4	0.0
Pork Shoulder									
50	49.1	50.1	2.8	39.6	38.3	10.6[a]	10.8	10.9	1.0
40	39.3	38.6	40.1[a]	47.4	47.6	1.1	13.2	13.1	0.8
30	29.5	29.8	2.8	54.8	54.4	2.5	15.0	14.9	1.3
20	19.5	19.7	0.6	62.6	62.5	0.5	17.1	17.3	4.4
Overall mean	34.4	34.5	0.9	51.1	50.7	9.4[a]	14.0	14.0	0.0
Beef Shank, Cheek, and Tongue									
Shank 1	4.0	4.1	1.7	74.1	74.2	0.5	21.0	21.0	1.1
2	3.8	4.0	2.0	74.2	73.8	12.3[a]	21.3	22.0	28.8[a]
Overall mean	3.9	4.1	3.7	74.2	74.0	4.8[a]	21.2	21.5	22.0[a]
Cheek 1	12.3	11.5	10.6[a]	67.9	68.7	97.2[a]	19.2	19.8	28.1[a]
2	14.3	13.3	34.2[a]	66.3	66.7	5.2	19.1	19.1	0.0
Overall mean	13.3	12.4	38.0[a]	67.1	67.7	41.2[a]	19.2	19.5	11.8[a]
Tongue 1	18.9	19.5	6.0	64.2	64.0	1.3	15.9	15.7	6.7[a]
2	15.5	16.1	9.2[a]	66.8	66.9	0.2	17.1	16.9	1.6
Overall mean	17.2	17.8	14.4[a]	65.5	65.5	0.4	16.5	16.3	6.9[a]

[a] Exceeds tabular *F*-value at $P = 0.05$.

high and low differences for these 2 meats suggest an inconsistent or random variation in the data rather than a constant difference resulting from a treatment effect. A large between-means difference is considered to be >1.2% fat [3 σ value of repeatability established in a collaborative study of **24.005(a)** (6)].

Analysis of variance of moisture determinations (Table 5) indicated that 3 paired means of the 5 meat types differed significantly but not all in the same direction, suggesting an inconsistent variation in the data rather than a treatment effect. The largest difference between means, 0.6% (cheek meat), was considered to be an acceptable level of difference. A large between-means difference would be >0.9% moisture [3 σ value of repeatability established in a collaborative study of **24.003** (7)].

Analysis of variance of protein determinations (Table 5) indicated that 3 paired means of the 5 meat types differed significantly, again not all in the same direction, suggesting an inconsistent variation rather than a treatment effect. Mean differences are all within 0.3% protein and this is considered an acceptable level of difference. A large between-means difference would be >0.6% protein [3 σ value of repeatability established in a collaborative study of **24.027** (8)].

Conclusion

Examination of variance of replicate determinations of fat, moisture, and protein determinations on 5 types of meat, with preparation treatment as the principal variable, showed that precision of determination was higher for samples prepared with a bowl cutter than with a food chopper. This observation suggested that samples prepared with a cutter were more homogeneous than those ground with a chopper. Complete and reproducible homogeneity would involve more elaborate conditions than those used here. Prechilling, essential to a meat sample preparation procedure, provided firmness of fat and minimized loss of sinewy tissues and meat juices during cutting or grinding operations.

Analysis of variance of replicate determinations of samples treated with either bowl cutter or chopper procedures indicated that accuracy between the 2 procedures was equivalent for fat,

moisture, and protein content. Differences between the means were random without an indication of treatment effect and were within acceptable tolerance for each method of proximate analysis.

Overall analyses on meat samples indicated the bowl cutter to be an adequate alternative to a food chopper. Advantages include convenience, simplicity, analysis of a prepared sample without an additional mixing step, and homogeneity of a prepared sample for good analytical precision. It is recommended that the procedure be adopted as an alternative to **24.001** for meat analysis.

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FERTILIZERS

Determination of Magnesium, Iron, and Zinc in Fertilizers by Flame Atomic Absorption and by Inductively Coupled Plasma Emission Spectroscopy: Comparison of Methods

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Determination of Mg, Fe, and Zn by inductively coupled plasma emission spectroscopy (ICP) and by the official flame atomic absorption spectrophotometric (FAA) method were compared. Samples were acid digests of commercial fertilizers. Neither matrix effects nor spectral interferences were encountered in the ICP analyses. Linear regression shows that the slopes obtained by plotting ICP results vs FAA results for each element differ from unity by less than 5%.

Elemental analysis by using emission spectra from plasma excited by an inductively coupled rf generator (ICP) or by a dc arc is a relatively new variation of an old method (1). Modern ICP spectrometers are suitable for rapid, multi-element analysis of solutions, and have a sensitivity comparable to that of flame atomic absorption (FAA) but with somewhat less specificity. Because of its speed, it is desirable to test this method on a variety of samples to see where, among standard methods, it may find a place.

Studies of some environmental materials have been reported, for example, marine sediments (2), soils, and plant materials (3, 4). Elemental analyses of fertilizers by dc plasma emission have been reported by Hunter, Woodis, and others who determined aluminum, iron, calcium, magnesium, and uranium in phosphate rock and wet-process phosphoric acid (5, 6), and boron and molybdenum in mixed fertilizers (7, 8). We offer here a direct comparison of results obtained by ICP and by FAA for magnesium, iron, and zinc in fertilizers. At this time there is not a substantial body of experience with ICP analyses. This work is a preliminary step toward further evaluation of the method for fertilizers.

Experimental

Samples were selected from commercial fertilizers sent to this laboratory by New York State Inspectors for regulatory analysis. Solutions were prepared for atomic absorption analysis by the AOAC official method (9). Briefly, this procedure is as follows: Dissolve sample in HCl, boil to dryness, dissolve the residue in dilute HCl, filter the solution, dilute and add enough lanthanum stock solution to make the final solution 1% La, and measure absorption. The same solutions of both samples and standards were used with both instruments. The standards were prepared by quantitative dilution of commercial reference solutions (Fisher Scientific Co.).

The atomic absorption spectrometer used was a Perkin-Elmer Model 305B operated with an acetylene-air flame. Duplicate readings were averaged for each analysis. The ICP spectrometer was Instrumentation Laboratory Model Plasma 100. Each determination was an average of 5 readings taken in sequence during a single aspiration of the sample solution. Emission wavelengths were 279.1, 238.2, and 213.9 nm for Mg, Fe, and Zn, respectively.

Results and Discussion

The experimental results are listed in Table 1. The principal components of each fertilizer are given to show the variety of sample compositions used. The differences between ICP and FAA results do not correlate with the fertilizer compositions. This is not surprising because the solutions were quite dilute, less than 0.06% dissolved solids in addition to the 1% La, and the differences were always small.

Some statistical parameters which illustrate the agreement between the ICP and FAA results are given in Table 2. If there were no errors in the data, each pair of measurements should be identical. An easy way to test this condition is to plot a graph of ICP results against FAA results. The graph should be a straight line of unit slope passing through the origin:

$$(\mathrm{ICP}) = m(\mathrm{FAA}) + b$$

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Table 1. Paired determinations of Mg, Fe, and Zn in fertilizers by ICP and FAA [a]

	Mg			Fe			Zn	
Fertilizer [b]	ICP	FAA	Fertilizer	ICP	FAA	Fertilizer	ICP	FAA
0- 5-28	7.59	7.24	7-39-12	2.87	2.73	12-11-14	0.953	0.918
6- 9-10	5.64	6.03	19- 4- 6	2.09	2.00	11-12-15	0.931	0.907
8- 8- 8	5.24	5.15	9- 4- 3	1.83	1.86	11- 9-14	0.782	0.762
4-12- 6	5.00	4.87	12-20- 9	1.73	1.68	13-14-14	0.760	0.734
8- 8- 8	4.55	4.75	27- 0- 6	1.44	1.43	15-15-14	0.529	0.519
5- 8-17	4.10	4.07	27- 4- 5	1.38	1.31	12-23-12	0.403	0.397
5- 8-17	4.07	4.03	27- 3-11	0.810	0.801	12-23-12	0.334	0.330
8-16-16	3.91	3.78	22- 4-13	0.732	0.764			
12- 5- 9	3.58	3.76	13- 6- 6	0.458	0.489			
20- 4- 8	2.98	3.16	21- 0-11	0.474	0.465			
6-20-20	3.01	2.98	8- 4-15	0.233	0.239			
11-22-15	2.88	2.94	8- 4-15	0.182	0.185			
10-20-11	2.35	2.42						
10-20-11	2.38	2.31						
10-20-11	2.24	2.30						
11-20-11	2.32	2.24						
10-19-12	2.09	2.15						
10-16-16	2.09	2.11						
10-20-11	2.03	2.09						
9-16-16	1.54	1.56						
19- 5- 9	0.704	0.719						
12-23-21	0.705	0.710						
12-23-21	0.554	0.538						
18-18-22	0.396	0.415						
5- 9- 9	0.287	0.312						

[a] Weight percent in the fertilizer.
[b] Weight percent N, P_2O_5, and K_2O, respectively.

In the absence of error, $m = 1$ and $b = 0$. The slope, m, and intercept, b, are shown in Table 2 for the straight line fit to each set of data by a least squares calculation. The slopes are within 5% of unity and the intercepts are approximately 1% of the maximum concentration. The scatter of the points about the lines may be shown in 2 ways, by the coefficient of variation and by the average difference between ICP and FAA measurements. The square of the coefficient of variation is greater than 0.99 in each case (Table 2). The average difference, ⟨ICP − FAA⟩, should be zero if there is no systematic error, and the standard deviation of this quantity provides a measure of the random error. Both the relative average differences and their standard deviations are within 4% of zero. An analysis of variance for the relative errors is shown in Table 3. The F_s value falls between those for 95 and 99% certainty in rejection of the hypothesis that variance among elements is important. Most of the variance comes from the results for zinc where it appears that a small systematic error may have occurred.

Table 2. Statistical parameters for the correlation of determination by ICP with FAA

Element	n	m [c]	$b \times 10^2$ [c]	r^2 [d]	⟨Difference⟩ × 100 [e] ± std dev.
Mg [a]	25	1.008	−3.86	0.9944	−1.08 ± 3.49
Mg [b]	16	0.997	0.32	0.9978	
Fe [a]	12	1.049	−3.4	0.9980	0.45 ± 3.77
Fe [b]	16	1.018	−2.12	0.9974	
Zn [a]	7	1.046	−1.2	0.9999	2.47 ± 0.98
Grand av. [a]					−0.10 ± 3.51

[a] This study.
[b] Hunter et al. (5)
[c] Straight line fit by least squares: (ICP) = (FAA)m + b
[d] Coefficient of variation squared.
[e] Average, relative difference: $\langle \text{Difference} \rangle = \frac{1}{n} \sum \frac{(\text{ICP})_i - (\text{FAA})_i m - b}{(\text{ICP})_i}$

Table 3. Analysis of variation with respect to elements

Source of variation	Degrees of freedom	Sum of squares	Mean square	F_S [a]
Elements	2	73.95[b]	36.98	3.33[*]
Residual	41	454.81	11.09	
Total	43	528.79	12.30	

[a] Values for F^2_{41} are 3.23 (5%) and 5.17 (1%).
[b] The individual element contributions to this term for Mg, Fe, and Zn are 24.21, 3.66, and 46.09, respectively.
[*] Significant at $P = 0.05$.

Errors in the measurements are several percent as described above and shown in Table 2. One source of error which is sometimes a difficult problem is incomplete recovery of analyte. For these samples, the question of recovery from the fertilizer samples was answered satisfactorily by earlier investigators who devised the official method of sample solution preparation. The only concern here is a comparison of recovery from the sample solutions because the same sample and standard solutions were used with both instruments. The average, relative difference values in Table 2 show that recovery from the sample solutions was essentially identical for the 2 instruments and that the errors in the measurements are primarily random. Our results for Mg and Fe are directly comparable to those of Hunter et al. (5) and show similar agreement (Table 2).

Although we did not take advantage of the analytical speed of the ICP spectrometer in this study, it is an obvious and attractive feature of the instrument, offering the possibility of rapid, comprehensive analysis of fertilizers. We intend to determine how well such fertilizer analyses can be carried out by ICP and recommend that a collaborative study in this area be initiated.

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High Pressure Liquid Chromatographic Determination of Nitrogen Derived from Urea and Water-Soluble Methylene Ureas in Urea Formaldehyde Fertilizers: Collaborative Study

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A method for measuring nitrogen derived from urea, methylenediurea (MDU), and dimethylenetriurea (DMTU) was tested by 9 collaborators. Collaborators made single determinations on 2 separate days on 5 sets of paired samples. Samples were extracted with water, filtered, and analyzed after high pressure liquid chromatographic separation on a C-8 or C-18 bonded silica column, using a refractive index detector. Peak heights compared to external standard of each component were used for quantitation. Mean coefficients of variation for the completed study were 3.24% for urea, 3.48% for MDU, and 6.56% for DMTU. The method has been adopted official first action.

Although urea formaldehyde (UF) fertilizers have been in the marketplace for over 20 years, the only method of characterizing them has been the Nitrogen Availability Index (NAI) (1). This analysis does not describe the water-soluble nitrogen of UF fertilizers, which comprises a large portion of many turf-type fertilizers. In addition, it has been shown that the hot water-insoluble nitrogen present is of limited value agronomically because it is only available over extended periods of time (2).

At least 2 companies have petitioned the Association of American Plant Food Control Officials (AAPFCO) for a definition change of slow-release nitrogen in UF fertilizers to include the water-soluble UF polymers present. To define the water-soluble fraction of UF fertilizers, a high pressure liquid chromatographic (HPLC) method was developed and subjected to collaborative study. The method measures the 3 major components of the water-soluble nitrogen fraction [urea, methylenediurea (MDU), and dimethylenetriurea (DMTU)], which usually account for up to 95% of the nitrogen present in that fraction.

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The recommendation of the Associate Referee was approved by the General Referee and Committee A and was adopted by the Association. See the Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, 329; 409.

Collaborative Study

Nine collaborative laboratories were furnished with 10 samples (5 matched pairs) and asked to perform a single analysis on each sample on 2 different days, using solutions of samples prepared on that day. The method; external standards of urea, MDU, and DMTU; a practice sample; and report forms were furnished to each collaborator. Collaborators were instructed to use peak height measurements for calculations and to return all data and chromatograms.

MDU and DMTU used as external standards were obtained by preparative HPLC. Identity was confirmed by elemental analysis, molecular weight, and infrared spectroscopy. The urea was Baker Analyzed Reagent grade used as purchased.

Pretest of the method.—Before the method was submitted to the collaborators, bonded reverse phase columns from various manufacturers were evaluated. Some differences in resolution and peak shape were experienced. Columns found to be acceptable were Apex ODS 5 μm, Jones Chromatography; μBondapak C-18, Waters Associates, Inc.; LiChrosorb RP-8, Merck; and Partisil 5 ODS-3, Whatman. Table 1 lists the columns used by the collaborators. A typical chromatogram is shown in Figure 1. Methylolureas such as monomethylolurea and dimethylolurea, if present, would have retention times between urea and MDU.

The effects of sample size, shaking time, and ultrasonic agitation were also determined. Completeness of extraction was evaluated by extracting sample sizes of from 1 to 10 g. The percent nitrogen due to urea, MDU, and DMTU for each of the samples was essentially the same. Urea nitrogen values were compared to the AOAC urease method (3).

The collaborative study samples were analyzed in our laboratory using the conditions described in the method. Analyses were run on 4 different days with fresh samples prepared each day. The same standards were used all 4 days. Mean

Table 1. Columns used by collaborators

Coll.	Column
1	μBondapak C-18
2	Hibar II LiChrosorb RP-8
3	Radial compressed module C-18, 5 μm
4	Regis Hi Chrom, reversible flow C-18, 5 μm
5	μBondapak C-18
6	Partisil PXS, 5/25 ODS
7	Radial compressed module C-18, 10 μm
8	Radial compressed module C-18
9	Hibar II LiChrosorb RP-8

coefficients of variation for the 5 matched pairs were 3.6, 5.9, and 7.8% for urea, MDU, and DMTU, respectively.

Samples.—The sample pairs analyzed in the collaborative study were selected to represent a range of UF ratios of from 1.3 to 2.5. The amount of water-soluble nitrogen (urea, MDU, and DMTU) is dependent on the UF ratio used in the production process. The fertilizer grade for each of the 5 matched pairs of samples was: samples 1 and 9 (38-0-0), samples 2 and 7 (27-3-3), samples 3 and 6 (34-4-4), samples 4 and 10 (26-3-3), and samples 5 and 8 (40-0-0). The study was designed to follow Youden's procedure for closely matched pairs (4).

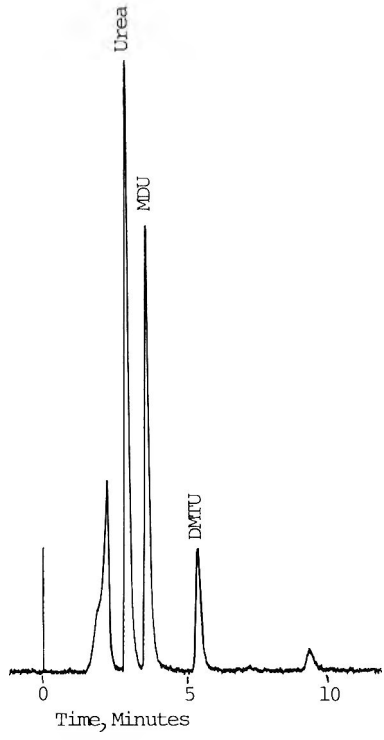

Figure 1. Typical chromatogram of the water-soluble fraction of urea formaldehyde fertilizer.

Urea and Water-Soluble Methyleneureas in Fertilizers

Liquid Chromatographic Method

Official First Action

2.D01 ***Principle***

Sample is ground to pass 40 mesh sieve, extd with H_2O, and filtered. Urea, methylenediurea (MDU), and dimethylenetriurea (DMTU) are detd by liq. chromatogy using external stds and refractive index detection.

2.D02 ***Apparatus***

(a) *Liquid chromatograph.*—With refractive index detector and pump capable of delivering mobile phase at 2 mL/min at pressures up to 2000 psig. Operating conditions: flow rate 1.0 mL/min (1500 psi); attenuator 8×; ambient temp.; injection vol. 10 μL. Sample injector with fixed sample loop preferred.

(b) *Chromatographic column.*—Partisil 5 ODS-3, 4.6 mm id × 25 cm (Whatman, Inc., 9 Bridewell Pl, Clifton, NJ 07014; other manufacturers' small particle reverse phase columns may be substituted with adjustments in operating conditions).

(c) *Strip chart recorder.*—Range to match output of detector.

2.D03 ***Reagents***

(a) *Mobile phase.*—HPLC grade H_2O.

(b) *Purified methylenediurea (MDU) and dimethylenetriurea (DMTU).*—Ext 50 g N-only ureaformaldehyde (UF) fertilizer with acetone 8 h on soxhlet extractor. Select UF fertilizer with high MDU/DMTU-to-urea ratio. Remove thimble from extractor, let air-dry, and collect residue. Mix 30 g acetone-washed residue in 300 mL H_2O and filter or centrf. Inject 100 mL supernate onto Waters Associates PrepPak 500 C-18 cartridge (5.7 × 30 cm) in preparative liq. chromatograph (Waters Associates Inc. Prep-500, or equiv.) at ambient temp. and with H_2O mobile phase at 150 mL/min. Collect top third of MDU and DMTU peaks. Evap. collected fractions to dryness in hood, using heat lamps. Dry using vac. over P_2O_5. Confirm identity using anal. liq. chromatogy and elemental analysis: mp of purified material, detd in pyrex, should be 205–207°d for MDU and 231–232°d for DMTU.

(c) *External std solns.*—(*A*) Accurately weigh ca 1.0 g each of urea (Baker Analyzed Reagent) and purified MDU, transfer both weighed

compds to same 100 mL vol. flask, and dil. to vol. with H_2O. (*B*) Accurately weigh 0.0125, 0.025, 0.050, and 0.10 g purified DMTU into sep. 50 mL vol. flasks. (*C*) Pipet 2, 5, 10, and 15 mL of mixed urea/MDU stds (*A*) into the vol. flasks from (*B*), resp. Dil. to ca 40 mL with H_2O and warm as necessary to dissolve DMTU. Cool to room temp. and dil. to vol. Approx. std contents = (*1*) 0.25 mg DMTU + 0.4 mg urea/MDU per mL; (*2*) 0.50 mg DMTU + 1.0 mg urea/MDU per mL; (*3*) 1.00 mg DMTU + 2.0 mg urea/MDU per mL; (*4*) 2.00 mg DMTU + 3.0 mg urea/MDU per mL.

2.D04 *Preparation of Sample*

Grind sample to pass 40 mesh sieve. Accurately weigh 2.000 g well mixed ground sample into 200 mL vol. flask. Add 150 mL distd or deionized H_2O, place on wrist-action shaker 20 min, and dil. to vol. with H_2O. Using glass fiber paper, filter portion into 4 mL vial. Filter again thru 0.45 μm filter before injection.

2.D05 *Determination and Calculations*

Inject 10 μL of each mixed std until peak hts agree ±2%. Inject 10 μL sample. Repeat stds after all samples have been injected. Std peak hts should agree within 3% of initial std peak hts. Average peak hts for each component and plot mg/mL vs peak hts.

$$\% \text{ Urea N} = \text{mg/mL (from graph)} \times 9.33/\text{g sample}$$

$$\% \text{ MDU N} = \text{mg/mL (from graph)} \times 8.484/\text{g sample}$$

$$\% \text{ DMTU N} = \text{mg/mL (from graph)} \times 8.236/\text{g sample}$$

Results and Discussion

Collaborative results for nitrogen derived from urea, MDU, and DMTU are given in Tables 2, 3, and 4, respectively. The results of Collaborator 8 for urea fall outside the range of expected val-

Table 2. Collaborative results for HPLC determination of urea nitrogen

	Sample No.									
	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5	
Coll.	1	9	2	7	3	6	4	10	5	8
					Day 1					
1	4.55	4.31	8.46	9.79	11.58	11.31	8.61	9.07	10.48	10.34
2	5.08	4.59	8.28	9.51	11.71	10.86	8.82	9.16	9.89	10.09
3	4.63	4.26	8.67	9.59	11.70	11.00	8.71	8.86	10.10	10.10
4	4.59	4.54	8.43	9.05	11.22	11.06	8.77	8.92	10.15	10.23
5	4.55	4.32	9.24	9.80	12.40	11.60	9.21	9.45	10.50	10.40
6	4.70	4.42	8.66	9.57	11.62	11.16	9.02	8.98	10.25	10.25
7	4.57	4.24	8.48	9.37	11.52	10.63	8.21	8.86	9.84	10.01
8[a]	4.67	4.63	9.47	10.27	12.40	11.86	9.19	9.71	10.95	11.17
9[b]	4.65	4.30	8.90	9.62	11.88	10.99	8.74	8.84	9.91	10.02
					Day 2					
1	4.62	3.95	8.78	9.32	11.60	10.75	8.68	8.71	10.00	9.77
2	4.88	4.11	8.35	9.05	11.36	10.42	8.35	8.39	9.83	9.97
3	4.40	4.16	8.05	9.11	10.80	10.60	8.00	8.43	9.90	10.00
4	4.64	4.07	8.38	9.27	11.02	10.56	8.56	8.68	9.80	9.85
5	4.43	4.00	8.86	9.75	11.70	11.30	9.00	9.05	10.20	10.20
6	4.50	4.42	8.70	9.89	11.89	11.12	9.11	8.96	10.20	10.47
7	4.66	4.24	8.53	9.56	11.71	10.96	8.86	8.72	10.04	10.21
8[a]	5.03	4.52	9.18	10.26	12.25	11.79	9.32	9.61	10.99	10.86
9[b]	4.65	4.21	8.76	9.48	11.77	11.18	8.92	8.81	10.03	9.95
N	14		14		14		14		14	
Mean	4.44		9.02		11.26		8.79		10.11	
S_d	0.214		0.367		0.474		0.413		0.288	
S_r	0.147		0.174		0.193		0.166		0.099	
S_h	0.110		0.229		0.306		0.267		0.192	
$S = \sqrt{S_r^2 + S_h^2}$	0.184		0.287		0.362		0.314		0.216	
CV, %	4.1		3.2		3.2		3.6		2.1	

[a] Collaborator's results omitted by ranking test.
[b] Received late, results omitted.

Table 3. Collaborative results for HPLC determination of MDU nitrogen

	Sample No.									
	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5	
Coll.	1	9	2	7	3	6	4	10	5	8
					Day 1					
1	2.65	2.20	4.96	5.62	6.19	6.32	6.14	6.32	8.71	8.45
2	3.18	2.60	5.15	5.73	6.44	6.28	6.96[a]	6.64	8.47	8.45
3	2.63	2.31	5.24	5.65	6.09	6.11	6.09	6.36	8.31	8.15
4	2.65	2.30	5.22	5.38	6.20	6.49	6.32	6.52	8.58	8.53
5	2.67	2.01	5.43	5.43	6.45	6.19	6.36	6.32	8.69	8.38
6	2.66	2.39	5.28	5.60	6.30	6.22	6.30	6.49	8.40	8.27
7	2.71	2.29	5.30	5.55	6.36	6.19	6.06	6.45	8.27	8.27
8	2.89	2.44	5.22	5.71	6.05	6.41	6.24	6.64	8.90	8.85
9[b]	3.33	2.79	5.85	5.97	8.26	6.96	6.85	6.96	9.69	9.55
					Day 2					
1	2.73	2.11	5.25	5.40	6.25	6.13	6.14	6.10	8.35	7.93
2	3.13	2.31	5.30	5.72	6.44	6.24	6.09	6.20	8.58	8.37
3	2.69	2.27	4.91	5.38	5.71	5.94	5.71	6.00	8.22	8.11
4	2.76	2.17	5.13	5.49	5.96	6.15	6.13	6.23	8.22	8.09
5	2.63	2.37	5.26	5.68	6.53	6.28	5.98	6.40	8.44	8.52
6	2.60	2.23	5.30	5.75	6.56	6.45	6.42	6.38	8.46	8.20
7	2.80	2.25	5.17	5.60	6.28	6.23	6.19	6.19	8.37	8.27
8	2.88	2.54	5.30	5.60	6.38	6.32	6.06	6.70	8.90	8.74
9[b]	3.45	2.75	5.78	5.88	7.98	7.03	7.01	6.91	9.70	9.34
N	16		16		16		15		16	
Mean	2.53		5.40		6.25		6.25		8.42	
S_d	0.201		0.140		0.225		0.217		0.312	
S_r	0.111		0.118		0.139		0.142		0.090	
S_b	0.119		0.053		0.125		0.117		0.211	
$S = \sqrt{S_r^2 + S_b^2}$	0.162		0.130		0.187		0.183		0.230	
CV, %	6.4		2.4		3.0		2.9		2.7	

[a] Value exceeds 98% confidence limits; pair omitted.
[b] Received late, results omitted.

ues and were omitted as outliers based on the ranking test (4). One pair of nitrogen values for MDU and DMTU were omitted as outliers after applying the Dixon test (5) using 98% confidence limits.

The coefficients of variation for urea and MDU nitrogen are consistent for the 5 matched pairs of samples and are well within what would be expected for a method using external standards. The values for DMTU nitrogen are somewhat higher, probably due to the lower concentration of DMTU nitrogen present. The collaborative study gave mean coefficients of variation less than those initially obtained in our laboratory (see Table 5).

Collaborator 9 was not able to complete the collaborative study in time for inclusion in the statistical evaluation. Results reported were obtained using a UV detector at 202 nm and peak area measurements. Values for urea and DMTU are well within the range obtained by the rest of the collaborators. Values for MDU are considerably higher. In an attempt to determine the reason for this difference for MDU, a refractive index and UV detector (200 nm) were connected in series. A substantial difference was found in the amount of MDU detected with the 2 detectors, with the UV value being higher. To determine if an interfering peak was present, 2 columns, Whatman RAC ODS-3, 9.4 mm id × 10 cm, were connected in series. In this manner it was possible to separate MDU from a small peak on its tailing edge. Comparable results for MDU were then obtained for both detector systems. The extinction coefficient for the unknown peak must be quite high compared with its refractive index response to give essentially no apparent concentration with the refractive index detector. The small peak has not been identified, but Collaborator 9 did observe a peak with the same retention time as biuret in samples 1 and 9. He did not observe this peak in the remaining samples. Analysis of samples 1 and 9 with the column set up as above gave only the one peak ob-

Table 4. Collaborative results for HPLC determination of DMTU nitrogen

	Sample No.									
	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5	
Coll.	1	9	2	7	3	6	4	10	5	8
					Day 1					
1	0.91	0.91	2.32	2.31	1.80	2.06	2.84	2.98	5.39	5.03
2	1.09	1.07	2.30	2.26	1.86	2.08	3.08	3.21	4.98	4.98
3	1.03	0.97	2.26	2.21	1.85	2.21	3.04	3.13	5.13	5.01
4	0.94	0.98	2.12	2.25	1.78	2.13	2.98	3.03	5.24	5.05
5	0.95	1.13	2.10	1.96	1.96	2.18	2.72	3.09	4.94	4.42[a]
6	0.81	0.88	2.09	2.09	1.71	1.96	2.79	2.86	5.15	4.90
7	0.95	0.86	2.14	2.30	1.81	2.02	2.96	3.05	5.10	5.06
8	1.03	0.94	2.14	2.34	1.77	2.21	2.89	3.16	5.14	4.91
9[b]	0.90	0.88	2.33	2.27	1.82	2.10	2.94	3.05	4.99	4.90
					Day 2					
1	0.98	0.82	2.19	2.16	1.85	2.04	2.83	2.77	5.18	4.72
2	1.33	1.05	2.20	2.52	2.04	2.29	2.81	3.02	5.00	4.87
3	0.87	0.80	1.83	2.15	1.71	1.90	2.54	2.74	4.93	4.81
4	0.96	0.86	2.15	2.20	1.82	2.05	2.82	2.96	4.93	4.91
5	0.62	0.70	2.14	2.43	1.73	2.26	2.63	2.84	4.90	5.27
6	0.81	0.86	2.17	2.12	1.75	2.12	2.86	3.07	5.18	5.01
7	1.03	0.99	2.22	2.31	1.77	2.02	2.59	3.01	5.20	5.10
8	1.03	0.94	2.22	2.47	1.64	2.14	2.88	3.17	5.14	4.94
9[b]	0.96	0.88	2.25	2.24	1.73	2.10	3.03	3.03	5.02	4.84
N	16		16		16		16		15	
Mean	0.940		2.21		1.95		2.92		5.04	
S_d	0.171		0.150		0.123		0.189		0.134	
S_r	0.077		0.105		0.078		0.087		0.131	
S_b	0.108		0.076		0.067		0.119		0.019	
$S = \sqrt{S_r^2 + S_b^2}$	0.132		0.130		0.103		0.147		0.133	
CV, %	14.0		5.9		5.3		5.0		2.6	

[a] Value exceeds 98% confidence limits; pair omitted.
[b] Received late, results omitted.

served in all of the samples. The identity of this peak is yet to be established.

Collaborator 3 was concerned about the variability of his results, which he believed was due to increasing back pressure during his work on this method. He was able to show that peak heights were dependent on the back pressure of his system: As the back pressure increased, the peaks seemed to broaden giving rise to decreased peak heights. He also observed that back pressure tends to decrease slightly during a day of continuous operation. Typically, standards gave larger peak heights at the end of the day. Collaborator 3 believes that peak area rather than peak height would give more consistent results. Examination of the chromatograms of the other collaborators showed that peak heights of standard were, in general, slightly higher at the end of a run compared with initial values. This has also been noted in the Associate Referee's work but has not been a significant problem. None of the other collaborators gave any indication of problems with back pressure.

A larger injection volume (20–30 μL or greater) was suggested by Collaborator 4. This would allow the use of lower detector sensitivity, thus improving the signal-to-noise ratio. The overall result would be greater precision. Varying the sample size (up to 10 g) was included in the ruggedness test conducted by the Associate Referee. Repeated injections of 5–10 g samples

Table 5. Mean coefficient of variation for urea, MDU, and DMTU: comparison of in-house study with collaborative study results

	Av. CV, %	
Component	In-house	Collab. study
Urea	3.6	3.2
MDU	5.9	3.5
DMTU	7.8	6.6

with high levels of soluble constituents caused peak broadening with apparent loss of sensitivity, possibly due to column overload. A sample size of 2–4 g with an injection volume of 10 μL was determined to be optimum.

Collaborator 5 questioned the use of a DMTU standard higher than the solubility reported in the method. Water solubility for DMTU was determined on pure (99%+) material. Fertilizer samples analyzed by this method often contain DMTU levels which exceed the solubility determined, therefore, heating standards is specified to dissolve the larger quantity of DMTU.

The Associate Referee reported 10.21% urea nitrogen for the practice sample used in the collaborative study. Results reported by 3 collaborators and re-analysis of the sample here gave a lower value of 9.6%. Collaborator 8 had some problem with his urea standards and used the 10.21% urea nitrogen value to help establish a graph for urea; this was probably the reason for his high urea nitrogen results. The Associate Referee regrets any problem that this might have caused the collaborators.

Recommendation

The coefficients of variation obtained from the collaborative study are low and approach values obtained for many chromatographic methods using internal standards. Therefore, the Associate Referee recommends that the HPLC method collaboratively studied for determination of nitrogen derived from urea and water-soluble methylene ureas in urea formaldehyde fertilizers be adopted official first action.

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Barry Reese, Quality Assurance, O. M. Scott & Sons, Marysville, OH

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PRESERVATIVES

Gas-Liquid Chromatographic Determination of Benzoic Acid and Sorbic Acid in Foods: NMKL[1] Collaborative Study

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A gas-liquid chromatographic method for the simultaneous determination of benzoic acid and sorbic acid in foods was collaboratively studied by 8 laboratories. Benzoic and sorbic acids are isolated from food by successive extractions with ether, sodium hydroxide, and methylene chloride, converted to trimethylsilyl (TMS) esters, and determined by gas-liquid chromatography. Phenylacetic acid and caproic acid are used as internal standards for benzoic acid and sorbic acid, respectively. Seven samples were collaboratively studied: almond paste, fish homogenate, and apple juice with benzoic and sorbic acid levels from 0.04 to 2 g/kg. Average recoveries (%) for benzoic and sorbic acids were as follows: almond paste, 99.6 and 101.2; fish homogenate, 99.2 and 97.4; and apple juice 98.2 and 106.6. The reproducibility coefficients of variation (%) for benzoic and sorbic acids at 0.5-2 g/kg levels were 3.5-6.1 and 5.2-9.0; and at the 0.04 g/kg level, 14.7 and 23.3, respectively. The method has been adopted official first action at 0.5-2 g/kg levels.

Benzoic acid and sorbic acid are frequently used as preservatives in a variety of food products. The levels used normally range from 0.5 to 2 g/kg. Lower levels can occur in mixed food products as a result of carry over from ingredients. Natural occurrence of benzoic acid is reported in commodities such as berries (1) and dairy products (2, 3).

Official NMKL methods for determining benzoic acid (4) and sorbic acid (5) are based on gravimetry and/or titrimetry and colorimetry (thiobarbituric acid method), respectively. The benzoic acid method is not specific and other organic acids can interfere.

A previous method for the simultaneous determination of benzoic and sorbic acids by gas-liquid chromatography was published in 1974 (6) after being tested on a variety of foods. This method was improved and submitted to a NMKL collaborative study in 1978-79. The method used is given below.

Collaborative Study

Seven food samples were spiked with benzoic and sorbic acids at levels ranging from 0.04 to 2 g/kg. The samples, chosen to represent different food classes, were almond paste, studied at 2 levels; fish homogenate, 2 levels; and apple juice, 3 levels.

Eight laboratories from Sweden, Norway, Denmark, and Finland participated in this study. Each collaborator received 7 test samples and one practice sample with known concentrations of preservatives. The samples were to be stored frozen until analyzed. Along with the samples, sufficient amounts of standard preservatives and internal standards were enclosed.

Collaborators were instructed to analyze samples as duplicates, according to the proposed method. Samples containing <0.4 g/kg were to be re-analyzed, using a low level standard curve and with less internal standard added. The mixed standard solutions and the internal standard solution could be used for this purpose after proper dilution.

Participants were asked to report their results, including data on standard curves, i.e., coefficient of correlation, slope, and intercept, on a recording form. They were also requested to return one chromatogram of each sample.

Benzoic Acid and Sorbic Acid in Foods
Gas-Liquid Chromatographic Method
Official First Action
NMKL-AOAC Method

(Collaboratively tested on apple juice, almond paste, and fish homogenate (at 0.5-2 g/kg levels), representing carbohydrate-rich, pasty and rich

[1] Nordic Committee on Food Analysis (Secretariat General, c/o National Food Administration, Box 622, S-751 26, Uppsala, Sweden).

This method, with the modifications recommended by the author, was accepted as an official NMKL method at the 34th Annual Meeting of the Nordic Committee on Food Analysis, 1980.

The recommendation 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.* (1983) 66, 372; 421.

in fat and carbohydrates, and protein-rich foods.)

20.D05 *Principle*

Benzoic acid and sorbic acid are isolated from food by extn with ether and successive partitionings into aq. NaOH and CH_2Cl_2. Acids are converted to trimethylsilyl (TMS) esters and detd by GLC. Phenylacetic acid and caproic acid are used as internal stds for benzoic acid and sorbic acid, resp.

20.D06 *Apparatus*

(a) *Gas chromatograph.*—With linear oven temp. programmer, flame ionization detector, and recorder. Following conditions have been found satisfactory: column, 1.8 m × 2 mm (id) coiled glass with 3% OV-1 on 100–120 mesh Varaport 30. Operating temps: oven 80–210°, 8°/min; injection port 200°, detector 280°. N carrier gas 20 mL/min. Approx. retention times 2.5, 4, 5, and 6 min for caproic acid, sorbic acid, benzoic acid, and phenylacetic acid, resp.

(b) *Centrifuge.*—With head for 30 and 200 mL centrf. flasks.

(c) *Mechanical shaker.*—Bühler SM-B, or equiv.

20.D07 *Reagents*

Use anal. reagents thruout.

(a) *Internal std soln.*—Dissolve 250 mg phenylacetic acid and 250 mg caproic acid in 100 mL 3% aq. KOH soln.

(b) *Silylating agent.*—*N*-Methyl-*N*-trimethylsilyl-trifluoracetamide (MSTFA), available from Pierce Chemical Co, Rockford, IL, or Machery-Nagel Co, D-5160 Düren, GFR.

(c) *Std solns.*—Prep. mixed std solns in $CHCl_3$ of benzoic acid, sorbic acid, phenylacetic acid, and caproic acid, resp., of following concns:

1) 200, 200, 750, and 750 μg/mL
2) 400, 400, 750, and 750 μg/mL
3) 600, 600, 750, and 750 μg/mL
4) 800, 800, 750, and 750 μg/mL
5) 1000, 1000, 750, and 750 μg/mL

20.D08 *Preparation of Sample*

Homogenize sample in mech. mixer. If consistency of sample makes mixing difficult, use any technic to ensure that sample material will be homogeneous.

Received April 9, 1982.

20.D09 *Extraction*

(a) *General method.*—Accurately weigh 5.0 g homogenized sample into 30 mL centrf. tube with Teflon-lined screw cap. Add 3.00 mL internal std soln, 1.5 mL H_2SO_4 (1 + 5), 5 g sand, and 15 mL ether. Screw cap on tightly to avoid leakage. Mech. shake 5 min and centrf. 10 min at 1500 × *g*. Transfer ether layer with disposable pipet to 250 mL separator. Repeat extn twice with 15 mL ether each time.

Ext combined ether phases twice with 15 mL 0.5N NaOH and 10 mL satd NaCl soln each time. Collect aq. layers in 250 mL separator, add 2 drops of Me orange, and acidify to pH 1 with HCl (1 + 1). Ext with CH_2Cl_2, using successive portions of 75, 50, and 50 mL. If emulsion forms, add 10 mL satd NaCl soln. Drain CH_2Cl_2 exts thru filter contg 15 g anhydrous Na_2SO_4 into 250 mL r-b flask. Evap. CH_2Cl_2 soln in rotary evaporator at 40° just to dryness.

(b) *Cheese and food products with paste-like consistency.*—Accurately weigh 5.0 g homogenized sample into 200 mL centrf. flask. Add 15 mL H_2O and stir with glass rod until sample is suspended into aq. phase. Add 3.00 mL internal std soln, 1.5 mL H_2SO_4 (1 + 5), and 25 mL ether. Stopper flask carefully and check for leakage. Mech. shake 5 min and centrf. 10 min at 2000 × *g*. Transfer ether layer with disposable pipet to 250 mL separator. Repeat extn twice with 25 mL ether each time. Continue as in (a), beginning "Ext combined ether phases. . .".

20.D10 *Derivatization and Gas Chromatography*

Add 10.0 mL $CHCl_3$ to residue in 250 mL r-b flask. Stopper and shake manually 2 min. Transfer 1.00 mL $CHCl_3$ soln to 8 mL test tube with Teflon-lined screw cap and add 0.20 mL silylating agent. Cap and let stand 15 min in oven or H_2O bath at 60°. Inject duplicate 1 μL portions of sample soln into gas chromatograph. Start temp. program when solv. peak emerges. Measure peak hts and calc. peak ht ratios of benzoic acid/phenylacetic acid and sorbic acid/caproic acid. Use av. of duplicate ratios. Peak ht ratios for duplicate injections should differ ≤5%.

20.D11 *Preparation of Standard Curves*

Transfer 1.00 mL std solns to five 8 mL test tubes with Teflon-lined screw caps. Add 0.20 mL silylating agent to each tube, cap, and let stand 15 min in oven or H_2O bath at 60°. Inject duplicate 1 μL portions of std solns into gas

chromatograph. Use same conditions as for sample soln. Measure peak hts and calc. peak ht ratios of benzoic acid/phenylacetic acid and sorbic acid/caproic acid, resp. Peak ht ratios for duplicate injections should differ ≤5%. Plot wt ratios (x) vs av. peak ht ratios (y) for each preservative. Calc. slope and intercept of std curve by method of least squares.

20.D12 ***Calculation***

$$\text{Preservative, mg/kg} = [(y - a)/b] \times (W'/W) \times 1000$$

where b = slope of std curve; a = intercept; y = av. peak ht ratio of preservative/internal std; W = wt of sample in g; and W' = wt of internal std in mg.

Results

Results were received from all participants. Collaborator 2, however, did not report results on benzoic or sorbic acid for the low level (0.04 g/kg) apple juice sample because of difficulties with excessive background noise at high detector sensitivity settings.

The collaborative results for benzoic acid and sorbic acid are shown in Tables 1 and 2, respectively. Laboratory rank totals were calculated and the test for extreme results was applied (7). Sorbic acid results of Collaborator 6 were omitted from further calculations because they were outside the upper 2.5% points of distribution. Collaborator 8 had not followed the instructions for samples containing <0.4 g/kg and the reported benzoic and sorbic acid results on the low level apple juice sample, obtained by the general method, were excluded. Sorbic acid results of Collaborator 7 for the low level apple juice were excluded because the calculations were based on phenylacetic acid as internal standard instead of caproic acid.

Standard deviations (between laboratories, within laboratories, and overall), coefficients of variation (reproducibility and repeatability) and average recoveries were calculated for each sample and analyte (Tables 1 and 2).

A one-way analysis of variance showed that differences between laboratories were significant at the 95% level in the following cases: for benzoic acid at the 2 g/kg level in almond paste and at all levels in fish homogenate and apple juice; and for sorbic acid at the 1 g/kg level in almond paste and fish homogenate and at the 0.04 and 0.5 g/kg levels in apple juice.

A 2-way analysis of variance for the labora-

Table 1. Collaborative results (mg/kg) for determination of benzoic acid

	Almond paste		Fish homogenate		Apple juice		
Coll.	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2	Level 3
1	923	1852	499	2052	46	951	1027
	1018	1883	511	2083	48	952	1033
2	939	1956	514	1792	—[a]	883	921
	950	2113	526	1859	—[a]	912	976
3	1039	2139	519	2060	39	969	1029
	1042	2150	525	2074	39	969	1042
4	1042	1869	510	1973	37	992	1064
	1055	1908	544	1996	44	1007	1067
5	974	1896	421	2007	44	963	1031
	997	2003	449	2088	49	967	1036
6	888	1805	492	1981	51	909	969
	993	1953	512	2019	53	924	979
7	996	1985	495	2007	35	926	1013
	1015	2011	498	2028	36	936	1015
8	953	1947	493	2015	54[b]	927	1004
	956	1958	517	2121	57[b]	931	1094
Added, mg/kg	982	1987	501	2044	41	1001	1082
Av. rec., mg/kg	986	1964	502	2010	43	945	1019
SD, mg/kg							
Between labs	33	83	27	76	5.8	32	34
Within labs	36	62	14	40	2.6	9.4	27
Overall	49	103	31	86	6.4	33	43
Reproducibility CV, %	4.7	5.3	6.1	4.3	14.7	3.5	4.3
Repeatability CV, %	3.7	3.2	2.8	2.0	6.1	1.0	2.7
Av. rec., %	100.4	98.8	100.1	98.3	105.9	94.4	94.2

[a] Results not reported due to excessive detector background noise.
[b] Excluded from calculations because instructions on analysis at low levels were not followed.

Table 2. Collaborative results (mg/kg) for determination of sorbic acid

	Almond paste		Fish homogenate		Apple juice		
Coll.	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2	Level 3
1	815	1768	509	1038	43	500	487
	903	2069	523	1107	45	551	570
2	936	2093	514	1052	—[a]	500	536
	1008	2107	527	1105	—[a]	516	568
3	973	2054	458	939	46	497	540
	974	2082	466	946	48	497	551
4	1056	2078	426	1045	55	559	562
	1057	2123	548	1069	58	596	619
5	911	1863	416	915	29	500	562
	922	1930	417	945	34	505	566
6[b]	1118	2426	571	1023	51	594	617
	1163	2793	628	1133	69	661	684
7	1038	2094	527	1006	28[c]	566	583
	1137	2101	545	1022	30[c]	567	659
8	1007	2021	440	951	65[d]	524	549
	1024	2028	457	975	68[d]	540	574
Added, mg/kg	992	1963	502	1025	41	501	540
Av. rec., mg/kg	983	2029	484	1008	45	530	566
SD, mg/kg							
Between labs	74	65	27	60	10.2	29	18
Within labs	41	84	34	27	2.3	18	36
Overall	84	106	44	66	10.4	34	40
Reproducibility CV, %	8.5	5.2	9.0	6.5	23.3	6.4	7.0
Repeatability CV, %	4.1	4.1	7.0	2.6	5.1	3.4	6.3
Av. rec., %	99.1	103.4	96.4	98.4	109.1	105.8	104.8

[a] Results not reported due to excessive detector background noise.
[b] Data from this laboratory excluded after application of the rank test for outliers.
[c] Excluded from calculations because phenylacetic acid was used as internal standard instead of caproic acid.
[d] Excluded from calculations because instructions on analysis at low levels were not followed.

tory–level interaction showed a significant difference among laboratories at the 95% level for benzoic acid in almond paste, fish homogenate, and apple juice. The laboratory-level interaction for sorbic acid was not significant in any of the sample groups.

Figure 1 shows a gas chromatogram of an almond paste extract containing 1 g benzoic acid and 2 g sorbic acid/kg.

Collaborators' Comments

Participants reported no difficulties in carrying out the analyses according to the general method, i.e., for levels 0.4–2 g/kg. Several collaborators, however, reported difficulties on the low level (0.04 g/kg) apple juice sample: Collaborator 2, who was not able to obtain a low level standard curve because of excessive detector background noise, suggested that more sample should be weighed to maintain a good signal-to-noise ratio at low levels. The ether volumes in this case could probably be increased without changing the volumes of sodium hydroxide solution. Collaborator 5 indicated in his report that the detector background noise could constitute a problem at low levels. Collaborator 7 reported that caproic acid could not be used in calculation of the low sorbic acid level because of interference from the solvent peak. To reduce interference from background noise and solvent peaks, Collaborator 6 dissolved the low level sample residue in 2 mL chloroform before derivatization, instead of the prescribed 10 mL.

Two comments on the extraction procedure were made by Collaborator 5: Fish products should be centrifuged at speeds exceeding 3000 $\times g$ after extraction with ether; and a centrifuge taking 200 mL centrifuge bottles, as required for almond paste samples, is hardly ordinary laboratory equipment.

Collaborator 7 suggested that if one of the internal standards, i.e., caproic acid, could be omitted and phenylacetic acid used as internal standard for both benzoic and sorbic acids, calculations would be easier, specially when an integrator is used. Collaborator 7 also reported that the gas chromatographic peaks showed tailing, even for the standard solutions, and suggested that tailing would disappear if the percentage of liquid phase was increased to 9%.

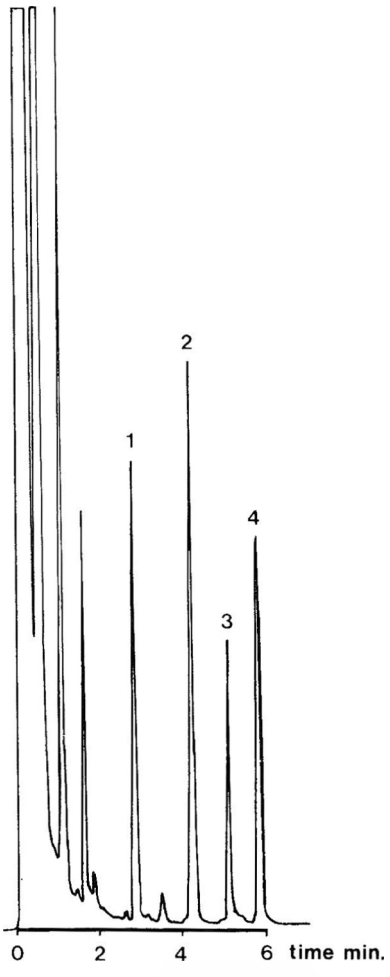

Figure 1. Gas chromatogram of almond paste extract showing TMS esters of caproic acid, internal standard (1); sorbic acid (2); benzoic acid (3); and phenylacetic acid, internal standard (4). Column 3% OV-1; temp. prog. 80-210°C, 8°/min.

Discussion

Some collaborators accepted rather large differences of peak height ratios between duplicate injections, which affect the repeatability and reproducibility. The method should be adjusted on this point, allowing peak height ratios of preservative/internal standard for each set of duplicate injections to differ by no more than 5%.

The poor reproducibility for benzoic acid and sorbic acid at 0.04 g/kg is an effect of measuring small peaks in the presence of interferences. To minimize this effect, the volume of chloroform needed to dissolve the extraction residue before derivatization should be reduced, as practiced by Collaborator 6.

The results of the study are on the whole satisfactory. The overall recovery from all samples was 98.9% for benzoic acid and 102.4% for sorbic acid. Although significant differences exist in the mean values between laboratories, the range of the mean values in the various samples is satisfactory and in line with other collaborative studies.

To study if phenylacetic acid could be used as internal standard for sorbic acid, as suggested by Collaborator 7, the participants were afterwards asked to recalculate the concentration of sorbic acid for each sample, on the basis of phenylacetic acid. The old chromatograms were used for this purpose, including the plotting of a new standard curve. The new sorbic acid results were sent to the Referee for evaluation. The results indicated that phenylacetic acid was more quantitatively extracted than sorbic acid, resulting in rather low recoveries. Using phenylacetic acid as internal standard, the overall recovery for sorbic acid from all samples was 89.1%. On the basis of these results, it seems favorable to maintain caproic acid as internal standard for sorbic acid.

Recommendations

It is recommended that the following modifications as suggested above be incorporated in the method: variation of peak height ratios of preservative/internal standard for each set of duplicate injections is limited to 5%; and the volume of chloroform for dissolving the extraction residue before derivatization is sufficiently reduced when low levels of preservatives are to be analyzed. Caproic acid should remain as internal standard for sorbic acid. The method with these few modifications should be adopted as an official NMKL method and an official AOAC method for the gas-liquid chromatographic determination of benzoic acid and sorbic acid in foods.

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FRUIT AND FRUIT PRODUCTS

Discussion of Statistical Methods for Determining Purity of Citrus Juice

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It is difficult to develop statistical methods to determine the purity of fruit juice because of country-to-country and environmental differences among fruit. A test of an unknown sample of citrus juice can err in 2 ways: Pure juice can mistakenly be rejected as adulterated, or adulterated juice can be accepted as pure. Many of the statistical procedures proposed may misclassify a high proportion of samples of pure juice as adulterated. It is necessary to develop a statistical test that will only rarely reject samples of pure juice, and will have a good chance of rejecting adulterated juice.

As in many other natural products, the composition of citrus juice is strongly affected by many factors. Concentrations of some of the components differ greatly among different fruit varieties. In addition, concentrations of components are affected by climatic, seasonal, and agrotechnical factors (1, 2). As a result of the wide variation among samples of pure fruit juice, norms or standards for the juice are described by ranges for each component. These ranges may be very wide so as not to reject samples of pure juice from varieties that have large or small concentrations of the component; consequently, they also do not reject samples of adulterated juice that are deliberately modified to conform to the acceptable ranges for pure juice. When the acceptable range is narrow, samples of pure juice may be rejected from varieties with extreme amounts of concentration for the component.

To differentiate pure and adulterated juices, investigators have proposed methods based on the relationships between the concentrations to 2 or more components. The simplest form of such a relationship is a ratio (3). However, the same problems that arise when using a range also arise when using a ratio. The range of permissible values for the ratio must be sufficiently large to pass juice of different varieties; otherwise, the use of a ratio not only discriminates between pure and adulterated juice of a specific variety, but also between pure juices of different varieties.

Rolle and Vandercook (4) propose using regression techniques to set limits for values of one characteristic based on limits for values of other characteristics. We show that regression limits are not applicable to samples of juice not taken under exactly the same circumstances as the series used in computing the regression limits.

Richard and Coursin (3, 5, 6) use a combination of many statistical techniques in trying to identify adulterated juice, but they do not indicate the chance of incorrectly rejecting pure juice as impure. As part of the series of tests, they use 95% (or less) confidence intervals for many characteristics. Ara and Török (7) show that multiple tests on one sample greatly increase the chance of *incorrectly* rejecting samples of pure juice. Richard and Coursin also use regression limits as proposed by Rolle and Vandercook. In addition, they propose 3 more sophisticated techniques: principal component analysis, correspondence analysis, and hierarchical classification. All these methods suffer from the same problem: They are applicable only to samples obtained under conditions similar to those initially taken.

There are difficulties in developing statistical tests that will be valid when applied to fruit juice. It is impossible to decide that juice is not pure based on a single sample, except in the most extreme cases. The proposed methods do *not* recognize that single samples may fail the test procedure due to random variation that occurs in pure juice.

We recommend procedures for testing new statistical tests before they are implemented.

Experimental

Sampling

Samples of orange juice were taken at random, by official inspectors, directly from the extraction

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machines, in citrus plants located throughout Israel. Sampling was carried out during 4 production seasons (i.e., 1976/77, 1977/78, 1978/79, and 1979/80). Each season started in November and ended in May. The samples were analyzed according to standard methods used for quality control of citrus juice (8).

Statistical Analysis

Regression coefficients and other statistical data were obtained by using the computer-aided statistical program BMDP9R (9).

Results and Discussion

Standards for One Characteristic

The simplest statistical approach is to determine acceptable limits for each characteristic, e.g., AFNOR (10) in France or RSK in Germany (11). Some of these limits are based on 95% confidence limits (12-14), that is, the limits describe an interval into which 95% of the samples of pure juice are expected to fall, and, on the average, 5% of the samples of pure juice will fall outside this interval.

The method based on 95% confidence limits assumes that random samples of pure juice were obtained from an underlying "given" normal population of pure juices. A new sample from the same "given" population will have a 5% chance that its value lies outside the limits. However, if the new sample is drawn from a different population, its value for the characteristic may lie outside the limits with far greater probability than 5% even when the sample is pure (2). Because distribution of the values of pure juice is a function of variety, maturity, and other environmental factors, the acceptable limits published may not be applicable to the juice being tested. More specifically, the acceptable limits should be computed from large multiyear samples of a specific variety and *only* then applied to that variety.

Testing Many Components

Usually the values of several characteristics from a single sample are assayed. Based on these values it is necessary to determine whether the juice is pure. One possible decision rule is that the juice is not pure if the value of any characteristic lies outside its 95% confidence interval. If, for instance, 5 characteristics are checked using this rule, there is a 22.6% chance that the value of at least one component is out of range (7). Using this rule, the chance of rejecting a sample increases as more characteristics are examined (40.1% and 64.2% for 10 and 20 characteristics, respectively).

To overcome this problem, statisticians have developed simultaneous test procedures (15) whose purpose is to limit the chance of rejecting a sample of pure juice to 5% (or some other percent), when more than one characteristic is tested. The simplest such method is to decrease the chance of being out of range for each characteristic to 5/K%, where K is the number of characteristics tested. To decrease the chance of being out of range, the length of the confidence interval must be increased.

If 5 characteristics are checked, the 95% confidence interval should be expanded to $\bar{x} \pm 2.6s$, where $\bar{x}$ is the mean and s is the standard deviation. For 10 characteristics, the 95% confidence interval should be $\bar{x} \pm 2.8s$, and for 20 characteristics, $\bar{x} \pm 3.0s$ (2).

Component vs Component – Simple Linear Regression

Let us assume that there is a linear relationship between the concentrations of 2 components. One of them (y) will be referred to as the dependent component and the other (x) as the independent component. Simple linear regression is used to find the coefficients a and b such that

$$\hat{y} = a + bx$$

where $\hat{y}$ is the predicted value of y when the value of x is observed. If the difference $y - \hat{y}$ is large, the observed value does not conform to the predicted value, and the juice will be called adulterated.

Figure 1 is a plot of potassium (mg/L) against ash (% w/w) for 66 samples of pure orange juice taken by government inspectors during the 1979/80 growing season. The 2 horizontal solid-shadowed lines represent the upper and lower limits of the acceptable range for potassium (95% confidence limits) obtained by computing its mean and standard deviation. A large area within the 2 lines does not contain any observations. Therefore, we would like to specify narrower bounds that would exclude the empty regions. One technique to limit the range of values for potassium is to specify the bounds as a function of ash, that is, the acceptable limits for potassium can be computed only after knowing the ash for the sample. The solid line that passes through the data is the regression line of potassium on ash. The 2 dashed lines are approximate 95% confidence intervals for observations about

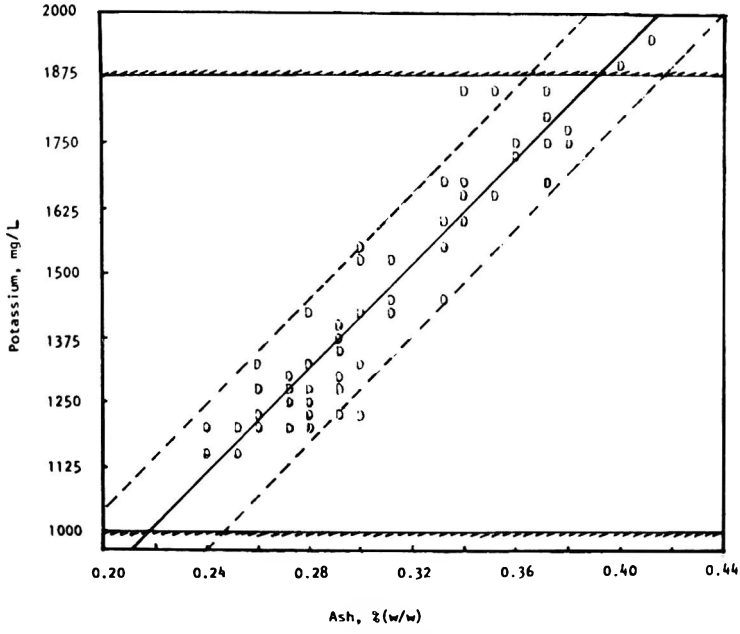

Figure 1. Potassium plotted vs ash for 66 samples of pure orange juice from 1979/80 growing season. Solid-shadowed horizontal lines represent upper and lower limits for potassium. Dashed lines are 95% confidence limits for observations about the regression line of potassium on ash (D = data point).

the regression line. These dashed lines restrict the possible range of values for potassium so that its acceptable range depends on the observed value of ash; these limits are more restrictive than the 2 horizontal lines.

Figure 2 is a plot of the same dashed lines, and a plot of 228 observations of pure juice taken during 3 other growing seasons (A = 1976/77, B = 1977/78, C = 1978/79). Note how many of the observations lie outside these dashed lines. That

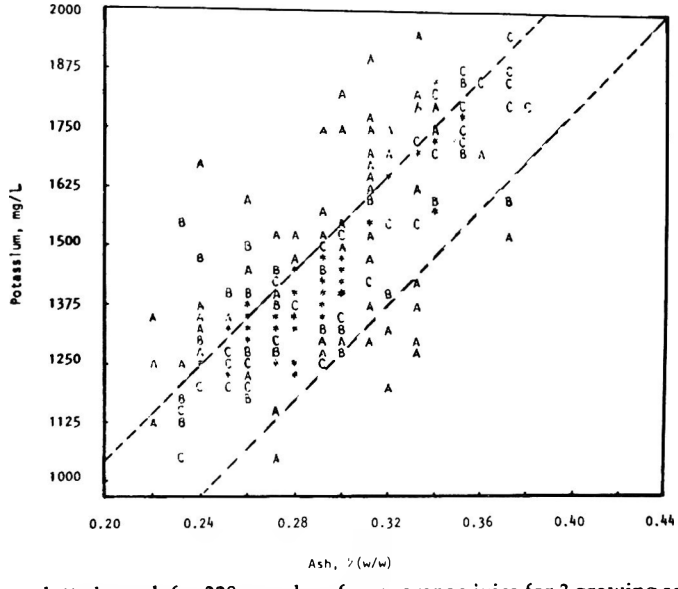

Figure 2. Potassium plotted vs ash for 228 samples of pure orange juice for 3 growing seasons. (A-1976/77, B-1977/78, C-1978/79, *overlap between 2 or more seasons).

Table 1a. Regression of potassium (mg/L) on ash (% w/w) for each of 4 growing seasons for Israeli orange juice

Growing season	No. of samples	Coefficients		Squared correlation (r^2)
		Constant	Slope	
1976/77	92	542	3189	0.23
1977/78	52	412	3545	0.56
1978/79	84	−55	5133	0.90
1979/80	66	−100	5064	0.90

is, the regression limits based on one growing season do not provide adequate quality control limits for data from other growing seasons.

The coefficients of the regression lines of potassium on ash for each of the 4 growing seasons are given in Table 1a. The last column (labeled R^2) is a measure of the quality of the fit of the regression line. (R^2 is the proportion of the variance of the dependent variable that is explained by the regression of the dependent variable on the independent variable(s).) Note the difference in the coefficients among the 4 seasons. A possible explanation for the difference is that the first 2 years were years of drought, whereas the last 2 were years of plentiful rain. It can be expected that the amounts of rainfall, or the lack of it, will affect the relationship between the components.

Table 1b shows the number of cases that exceed 1, 2, and 3 standard deviations from the regression line. If the differences $y - \hat{y}$ (observed minus predicted value) are normally distributed, then 31.7%, 5%, and 0.26% of the samples should fall outside these limits. Note the last 2 lines in the table. They correspond to using the regression lines for the 1978/79 and 1979/80 growing seasons. Both reject the data from the 2 earlier seasons at rates much higher than expected theoretically (52.2, 58.7, 19.2, and 21.2% instead of 5%). On the other hand, the regression line for 1976/77 does not reject too many samples from the other years. The last 2 growing seasons are years for which R^2 is very high, that is, the data are concentrated about the regression line. Therefore, the limits about the regression line are narrow. Hence, in a year when the relationship between the components changes (even slightly), there is a very good chance that the observed data will not conform to the norms based on the regression line. On the other hand, the regression line based on the 1976/77 growing season did not fit the data very well; therefore, the interval about the regression line is large. Hence, few observations from the other growing seasons were incorrectly rejected.

Component vs Component – Using Multiple Linear Regression

Rolle and Vandercook (4) propose that relationships between characteristics be used as a method of testing purity of fruit juice. Since the values of many characteristics rise or fall in parallel, Rolle and Vandercook suggest that the relationships be modeled by a regression equation of the form

$$\hat{y} = a + b_1x_1 + b_2x_2 + \ldots$$

where $\hat{y}$ is the value of one characteristic (called the dependent characteristic) predicted from the values of the related characteristics, $x_1, x_2, \ldots$, and $a, b_1, b_2, \ldots$ are coefficients of the linear regression of y on $x_1, x_2, \ldots$. Then the difference

$$y - (a + b_1x_1 + b_2x_2 + \ldots)$$

is an indication of how far the value of the first characteristic is from the value expected by the regression formula. The authors expect that this difference will be small when the juice is pure, and large otherwise.

Rolle and Vandercook (4), Richard (16), and Richard and Coursin (3) recommend using many characteristics on the right-hand side of the above equation so that the fit of the regression will be very good. This approach may cause a serious error.

Table 2a gives the coefficients of the regression line of potassium with brix, acidity, ash, and chloramine number (the independent variables). The number of cases that exceed 1, 2, and 3 standard deviations from the regression line are shown in Table 2b. We see that 80.4% of the samples from the 1976/77 growing season exceed one standard deviation from the regression line based on data from the 1979/80 growing season, and 54.3% exceed 2 standard deviations. Similar large discrepancies occur whenever the data are compared with the results of a regression line from another growing season.

Table 1b. Percent of samples that exceed 1, 2, and 3 standard deviations from regression line

Regression based on growing season	Samples tested from growing season											
	1976/77			1977/78			1978/79			1979/80		
	1SD	2SD	3SD	1SD	2SD	3SD	1SD	2SD	3SD	1SD	2SD	3SD
1976/77	29.3	2.2	2.2	7.7	0	0	6.0	0	0	13.6	0	0
1977/78	59.8	21.7	3.7	30.8	7.7	1.9	23.8	0	0	31.8	4.5	0
1978/79	79.3	52.2	27.2	46.2	19.2	9.6	38.1	3.5	0	56.1	18.2	3.0
1979/80	82.6	58.7	31.5	55.8	21.2	7.7	51.2	8.3	0	25.8	6.0	1.5

The analysis was repeated by using the regression of γ-aminobutyric acid on arginine only (Tables 3a and 3b), and multiple linear regression of γ-aminobutyric acid on 7 other amino acids (proline, alanine, valine, lysine, aspartic acid, arginine, and glycine). The results are shown in Tables 4a and 4b. (The data from 1977/78 growing season are not used because of the small number of samples examined in this growing season.)

Note that the higher the correlation R when estimating the coefficients, the narrower will be the limits about the regression line. For this reason both Rolle and Vandercook (4) and Richard and Coursin (3, 5, 6) recommend the use of regression models with large correlations. Unfortunately, if the regression model is not appropriate for the juice being tested, the pure juice will have a greater chance of falling outside the acceptable limits when R is larger than when R is small (since the acceptable limits are smaller in the first case). In almost all cases, the percent of rejections was far in excess of the percent that we expected to reject (31.7%, 5%, 0.26%). Therefore, criteria based on one growing season cannot apply directly to another growing season.

Critique of Proposed Statistical Methods

Richard and Coursin (3, 5, 6) propose multiple techniques for assessing the purity of fruit juice. Their approach consists of 2 stages. In the first stage, the values of the sample are compared with various standards and with results of previous samples, and a decision is made about the purity of the juice, e.g., that the juice is pure or that it is adulterated. If the decision is that the juice is adulterated, then in the second stage analysis they estimate the amount of water or ingredients added during adulteration. The decision-making stage (the first) is critical. Brown et al. (2) show that, when a sample of pure juice is tested, there is a very high probability (90% or higher) that the decision will be that the sample is adulterated. Therefore, a conclusion by their statistical method that the juice is adulterated is not proof that it is adulterated.

There are several reasons why the proposed techniques can reject pure juices far too often. Among the reasons are that multiple tests are performed (each having a 5% (or greater) chance of rejecting a sample of pure juice); effects of differences among countries, species, and climatic and environmental factors are not taken into account; and differences from season-to-season in the same species are not considered.

Table 2a. Regression of potassium (mg/L) on brix, acidity (% as citric acid), ash (% w/w), and chloramine number (mL chloramine-T, 0.01N/mL) for Israeli orange juice

Growing season	No. of samples	Coefficients					Squared multiple correlation (R^2)
		Constant	Brix	Acidity	Ash	Chloramine	
1976/77	92	−830.9	71.9	99.7	2426	56.6	0.41
1977/78	52	4.85	−0.218	249.0	3517	10.3	0.64
1978/79	84	−119.5	−3.08	50.5	5077	5.0	0.90
1979/80	66	−386.5	16.81	189.9	4217	10.7	0.92

Table 2b. Percent of samples that exceed 1, 2, and 3 standard deviations from regression line

Regression based on growing season	Samples tested from growing season											
	1976/77			1977/78			1978/79			1979/80		
	1SD	2SD	3SD	1SD	2SD	3SD	1SD	2SD	3SD	1SD	2SD	3SD
1976/77	25.0	3.3	2.2	11.5	0	0	10.7	0	0	18.2	0	0
1977/78	55.4	16.3	5.4	28.8	3.8	3.8	17.9	3.6	0	18.2	1.5	0
1978/79	78.3	44.6	22.8	42.3	17.3	5.8	36.9	5.9	0	51.5	16.7	1.5
1979/80	80.4	54.3	25.0	53.8	15.4	3.8	48.8	7.1	2.4	25.8	4.5	1.5

Table 3a. Regression of γ-aminobutyric acid (mM/L) on arginine (mM/L) for each of 3 growing seasons for Israeli orange juice

Growing season	No. of samples	Coefficients		Squared correlation (r^2)
		Constant	Slope	
1976/77	62	1.179	0.421	0.59
1978/79	82	0.618	0.645	0.72
1979/80	47	0.482	0.665	0.56

Table 3b. Percent of samples that exceed 1, 2, and 3 standard deviations from regression line

Regression based growing season	Samples tested from growing season								
	1976/77			1978/79			1979/80		
	1SD	2SD	3SD	1SD	2SD	3SD	1SD	2SD	3SD
1976/77	24.2	6.5	0	32.9	10.9	1.2	53.2	21.3	4.2
1978/79	48.4	14.5	3.2	24.4	2.4	1.2	61.7	17.0	2.1
1979/80	22.6	3.2	1.6	8.5	1.2	1.2	34.0	2.1	0

Table 4a. Regression of γ-aminobutyric acid (mM/L) on proline, alanine, valine, lysine, aspartic acid, arginine, and glycine (mM/L) for each of 3 growing seasons for Israeli orange juice

Growing season	No. of samples	Coefficients								Squared multiple correlation (R^2)
		Constant	Proline	Alanine	Valine	Lysine	Aspartic	Arginine	Glycine	
1976/77	62	0.56	0.133	−0.53	1.48	−0.48	0.011	0.18	3.62	0.85
1978/79	82	0.98	0.127	−0.57	0.08	0.70	0.02	0.18	2.26	0.85
1979/80	47	0.57	0.074	−0.84	3.78	2.22	0.26	0.13	0.89	0.79

Table 4b. Percent of samples that exceed 1, 2, and 3 standard deviations from regression line

Regression based on growing season	Samples tested from growing season								
	1976/77			1978/79			1979/80		
	1SD	2SD	3SD	1SD	2SD	3SD	1SD	2SD	3SD
1976/77	30.6	8.1	0	31.7	9.8	1.2	63.8	27.7	4.3
1978/79	45.2	1.6	0	29.3	3.7	1.2	57.4	25.5	2.1
1979/80	19.4	1.6	0	24.4	2.4	1.2	27.7	8.5	0

Quality Control

Two principle concepts in quality control are (a) producer's risk—the probability that a batch of pure juice will be rejected as adulterated. (b) Consumer's risk—the probability that a batch of adulterated juice will be accepted as pure. Ideally, both these risks should be zero.

Usually quality control is applied to products in which each item tested can be identified as defective or not. The risks then refer to the percentages of defectives allowable before rejecting the batch or lot. However, as long as the adulteration is produced by adding only characteristics that appear in the pure juice, there is no absolute method of differentiating pure and adulterated juice.

Therefore, the risks when applied to testing juice must refer to probabilities of correctly classifying the juice as pure or not pure.

We have shown above that there is great difficulty in modelling the distribution of characteristics of pure juice. In a test for adulterations, Lifshitz et al. (17) chose characteristics whose values had low coefficients of variation, gave reproducible and dependable results, had simple procedures for assaying, did not change over the entire season, and did not change with pasteurization or storage. These are minimal requirements for characteristics used in a quality control procedure.

The first concern of any procedure is to limit producer's risk. Falsely identifying pure juice as adulterated can cause severe monetary loss (including loss of reputation). Therefore, acceptable limits must be set to reject pure juice no more than 1% and preferably less than 0.1%. We point out that quality control limits are usually set at 3 standard deviations which corresponds to 0.26%. Our reason for using a more extreme limit is that the distribution of the values of a characteristic is not normal, as assumed by many statistical tests. Hence, more extreme values occur more often than is indicated by statistical theory. Setting a more extreme limit (such as 0.1%) compensates for the non-normal distribution.

Such a low value for producer's risk causes wide limits for accepting a sample as pure. In other words, the chance of rejecting a single sample of adulterated juice is poor and the consumer's risk is great. However, if more than one sample is assayed, the producer's risk remains the same, but the consumer's risk decreases. Hence, quality control procedures describe how many samples need to be assayed to achieve specified consumer and producer risks.

A major fault in many of the procedures proposed for citrus juice is that they presume to apply to a single sample. The procedures used for citrus juice should parallel procedures for other products in terms of quality control procedures except that the decision—pure or adulterated—is based on a statistical test.

Statistical Tests

It is possible to adapt available statistical methods to testing whether citrus juice is pure (17, 18). However, all methods require comparing a new sample to previously collected samples of pure juice (a base sample). To the extent that the base sample is not representative of the source from which the new sample is drawn, the test will have no validity. That is, the base sample should be (a) from the same variety, (b) selected at random over the entire growing season, (c) selected from several growing seasons, if not from the current season. This implies that the base sample for the test must be specific to the sample being tested and not a sample of juice from unknown sources taken in some previous year. Hence producers should cooperate in making available a base sample to the consumer.

A second problem is that the distribution of the values of the characteristic may not be appropriate to the assumptions of the statistical test. This can be verified by separating the base sample randomly into 2 parts. The first part is used as the base sample and the second is tested as if it consisted of samples of juice of unknown quality. If the test is reliable, the results will be similar to those expected by the statistical theory. Even with the above checks, if too many rejections are obtained in a given growing season, it may be indicative of changed environmental conditions rather than adulteration. Therefore, a new base sample may be needed.

It is necessary to develop a statistical test that will only rarely reject samples of pure juice, yet will have a good chance of rejecting samples of adulterated juice. We believe that such a test can be developed; however, it will be necessary to test several samples at one time in order to reach a conclusion whether juice is pure or adulterated.

Acknowledgments

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opinions of the authors and not necessarily those of the Ministry.

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PESTICIDE FORMULATIONS

Simultaneous Determination of Folpet, Piperonyl Butoxide, and Pyrethrins in Aerosol Formulations by High Pressure Liquid Chromatography

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A high pressure liquid chromatographic (HPLC) method is described for the simultaneous determination of folpet, piperonyl butoxide, and pyrethrins in aerosol pesticide formulations. The aerosol propellants are removed from the can and the remaining components are determined by HPLC, using an Ultrasphere 5 μm ODS reverse phase column and a UV detector at 254 nm. Recoveries for the 3 components are greater than 98%.

Folpet, *N*-[(trichloromethyl)thio]phthalimide, is a protective fungicide that is commonly used for the control of apple scab, rose mildew, rose black spot, and cherry leaf spot. It is also used for fungal control in paints and plastics. Piperonyl butoxide and pyrethrins are commonly used insecticide combinations. Formulations containing folpet, piperonyl butoxide, and pyrethrins are currently being evaluated for combined fungicidal and insecticidal activity. For both quality control and stability study purposes, it is of interest to be able to determine all 3 components in a formulated product.

Although numerous publications have described the determination of piperonyl butoxide and pyrethrins in various formulations, few of these formulations included folpet; in fact, there is little in the literature concerning the determination of folpet in formulations. Pomerantz et al. (1) used gas-liquid chromatography (GLC) on an XE-60 or QF-1 column for the separation and determination of captan, folpet, and captafol in crops. Bevenue and Ogata (2) also used an XE-60 column for the GLC separation of captan and folpet. A method for determining folpet by infrared spectroscopy is described in the *EPA Manual of Chemical Methods for Pesticides and Devices* (3). Zadrozinska (4) used thin layer chromatography and *Penicillum cyclopium* for the separation and detection of 8 pesticides including folpet residues in fruits and vegetables. Hoodles et al. (5) investigated the separation and UV detection of 30 pesticides; however, few quantitative data were presented.

Moreover, although these authors used a linear gradient elution of 25–75% acetonitrile in water on a 5 μm ODS-bonded silica column (Spherisorb ODS), the time set to increase from 25 to 75% acetonitrile was not stated. Carlstrom (6) investigated the separation of folpet from 17 pesticides by using normal phase HPLC, and although he conducted a collaborative test, this test was performed on samples containing folpet as the only pesticide. Quantitative data for the other pesticides investigated were not given.

The procedure described here allows the simultaneous determination of folpet, piperonyl butoxide, and pyrethrins in the presence of commonly used emulsifiers in aerosol formulations by reverse phase HPLC using acetonitrile–water (65 + 35) containing 0.005M pentanesulfonic acid as eluant.

METHOD

Reagents and Apparatus

(a) *Solvents.*—Acetonitrile, HPLC grade (Burdick & Jackson, from Alltech Assoc., Sydney, Australia). Ethyl acetate and methanol, Univar grade (Ajax Chemicals, Sydney, Australia). 0.25M 1-pentanesulfonic acid in glacial acetic acid (Waters Associates, Sydney, Australia).

(b) *Mobile phase.*—65% acetonitrile in water, 0.005M in pentanesulfonic acid. To 650 mL acetonitrile, add 350 mL water containing one vial pentanesulfonic acid solution.

(c) *Pesticide standards.*—Folpet (99.9% pure, Chevron Chemical Co., San Francisco, CA); piperonyl butoxide (99.8% pure); pyrethrins (50% solution in an aliphatic hydrocarbon solvent, Coopers Laboratories, Sydney, Australia).

(d) *High pressure liquid chromatograph.*—Waters Model 6000 A solvent delivery system, U6K injector, and Model 450 variable wavelength detector (Waters Associates). UV detector coupled to Linear Instrument Model 300 Series recorder (Activon Scientific Services, Granville, Austra-

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Table 1. Recovery of folpet, pyrethrins, and piperonyl butoxide from aerosol formulations [a]

Folpet			Pyrethrins			Piperonyl butoxide		
Added, %	Found, %	Rec., %	Added, %	Found, %	Rec., %	Added, %	Found, %	Rec., %
0.50	0.48	96.0	0.100	0.100	100.0	0.35	0.35	100.0
0.50	0.50	100.0	0.100	0.102	102.0	0.35	0.34	97.1
0.50	0.50	100.0	0.100	0.097	97.0	0.35	0.34	97.1
0.57	0.55	96.5	0.150	0.148	98.7	0.40	0.41	102.5
0.57	0.58	101.8	0.150	0.148	98.7	0.40	0.40	100.0
0.57	0.56	98.2	0.150	0.151	100.6	0.40	0.41	102.5
0.65	0.65	100.0	0.200	0.200	100.0	0.45	0.44	97.8
0.65	0.64	98.5	0.200	0.206	103.0	0.45	0.45	100.0
0.65	0.64	98.5	0.200	0.202	101.0	0.45	0.44	97.8
Av. rec., %		98.8			100.0			99.4
SD		1.84			1.83			2.12
Coeff. of var, %		1.86			1.83			2.13

[a] Each recovery value represents the mean of duplicate 25 μL injections.

lia). Column: Altex Ultrasphere reverse phase ODS, 5 μm, 25 cm × 4.6 mm id (Edwards Instrument Co., Sydney, Australia). Use 25 μL Hamilton syringe (Waters Associates) for injection. Chromatographic conditions: mobile phase, acetonitrile–water (65 + 35) containing 0.005M pentanesulfonic acid; flow rate, 2 mL/min; detector; UV at 254 nm and 0.1 AUFS; chart speed, 0.5 cm/min.

Preparation of Standard

Accurately weigh 0.1 g pyrethrins (50.0% solution) and 0.26 g piperonyl butoxide into separate 100 mL volumetric flasks and dilute each to volume with methanol. Into another 100 mL volumetric flask, accurately weigh 0.036 g folpet and dissolve in ca 10 mL ethyl acetate. To this folpet solution, transfer 10 mL of each of the pyrethrins and piperonyl butoxide solutions and dilute solution to volume with methanol. This working standard contains 0.005% pyrethrins, 0.026% piperonyl butoxide, and 0.036% folpet.

Preparation of Sample

Accurately determine gross weight of a full aerosol can and refrigerate the can 3 h at 4°C. Remove can from refrigerator and immediately pierce top to relieve gas pressure. Open top with can opener and place can in 40°C water bath to dispel any remaining propellants.

Using several portions of ethyl acetate, transfer residue in can to a 250 mL volumetric flask and dilute to volume with ethyl acetate. Dilute a 1 mL aliquot of this solution to 25 mL with methanol in a volumetric flask. Dry can and reweigh. Subtract this weight from gross weight to obtain net contents weight, which is also sample weight.

Determination

Using chromatographic conditions previously described, make duplicate 25 μL injections of both standard and sample solutions. Each set of duplicate injections should agree within ±2%. Calculate average peak areas (or heights) of the folpet and piperonyl butoxide peaks and the main pyrethrin peak for the duplicate injections of both standard and sample solutions. Calculate percentage of each component

$$\%\,(w/w) = (A'/A) \times C \times (25/100) \times 250 \times \%P \times (100/W)$$

where A and A' = peak areas (height) of standard and sample, respectively; C = concentration of standard (g/100 mL solvent); $\%P$ = percent purity of standard; and W = weight of can contents (g).

Apply the above relationship to the peaks corresponding to folpet, piperonyl butoxide, and pyrethrin, respectively.

Results and Discussion

Standard solutions of folpet, piperonyl butoxide, and pyrethrins were used to establish the chromatographic conditions necessary for separating these components and determining their retention times.

Because commercial pyrethrins consist of pyrethrins I and II, as well as lesser amounts of cinerin and jasmolin, the retention time as well as all subsequent calculations are based on the major earliest eluting pyrethrin peak.

The retention times of folpet, piperonyl butoxide, and pyrethrins (main peak) are 4.0, 7.2, and 10.0 min, respectively.

Aerosol cans were prepared in triplicate,

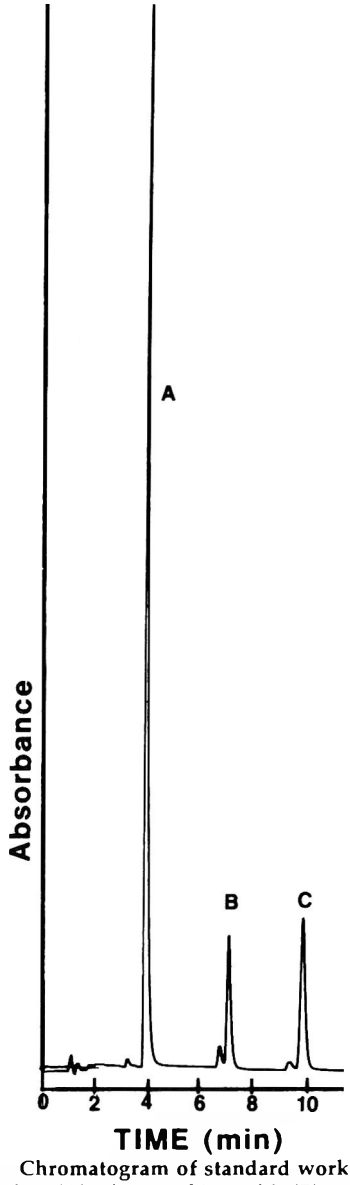

Figure 1. Chromatogram of standard working solution: folpet (A), piperonyl butoxide (B), main pyrethrins peak (C). Conditions as described in Reagents and Apparatus.

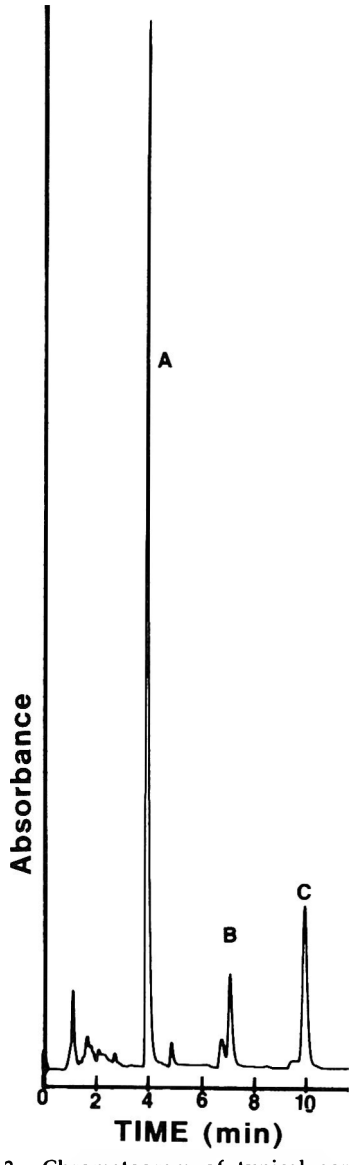

Figure 2. Chromatogram of typical aerosol can containing folpet (A), piperonyl butoxide (B), pyrethrins (C). Sample treatment and conditions as described in Method.

containing folpet, piperonyl butoxide, and pyrethrins in the concentrations shown in Table 1. The remaining can contents consisted of emulsifiers and propellant gas. Each can was analyzed by the method described above. Recoveries from the various cans are also shown in Table 1. Mean recovery over the concentration range studied for each component is folpet, 98.8%; pyrethrins, 100%; piperonyl butoxide, 99.4%. Figure 1 is a chromatogram of the working standard mix; Figure 2 is a chromatogram of the contents of a typical aerosol can treated as described above. As previously stated, when the concentration of pyrethrins is determined in the above formulation, the height of the main pyrethrin peak is used in the calculation

because pyrethrin extract consists mainly of esters of pyrethrolone (pyrethrins) and esters of cinerolone. As the relative amounts of ingredients in commercial extracts may vary, it is essential that the relative amounts of each component in the standard match those of the commercial extract used in the formulation if errors are to be avoided. We used the technical material actually used in the preparation of the aerosol cans as a standard for monitoring the composition of the can contents.

Conclusion

A relatively simple and accurate procedure has been developed for the simultaneous determination of folpet, piperonyl butoxide, and pyrethrins in aerosol concentrates and aerosol cans containing these components in addition to commonly used emulsifiers. In the concentration range studied, recovery of each component is greater than 98%.

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Reverse Phase Radial Compression High Performance Liquid Chromatographic Determination of Rotenone in Formulations

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This paper describes a reverse phase radial compression high performance liquid chromatographic (HPLC) method to determine rotenone in pesticide formulations. Formulations are extracted in dioxane by gentle swirling while sonicating for 5 min. A 10 μL aliquot is injected into the HPLC system equipped with a radial compression unit (Z module) containing a μBondapak C_{18} Radial-Pak column. The mobile phase is acetonitrile-methanol-tetrahydrofuran-water (34 + 30 + 1 + 35). Rotenone is monitored at 280 nm. Retention time for rotenone is approximately 7 min with total analysis time of 20 min. For 5 different products analyzed 11 times each, the percent coefficients of variation were all below 1.65. No interferences from 11 other rotenoids and 8 pesticides which are sometimes formulated with rotenone were observed.

The analysis of rotenone, a naturally occurring insecticide, in pesticide formulations by high performance liquid chromatography was first developed using normal phase (silica) chromatography (1). Because of the instability of silica columns, a reverse phase separation was developed in 1977 to analyze rotenone formulations (2). Elution time of rotenone using the normal phase and reverse phase methods was 12 and 17 min, respectively. In addition to the long analysis times, the extraction methods developed for each were lengthy.

This paper describes a reverse phase radial compression HPLC method that can be used to measure rotenone in pesticide formulations rapidly and accurately. Total analysis time, including sample preparation and extraction, is approximately 20 min.

METHOD

Apparatus and Reagents

(a) *High performance liquid chromatograph.*—Waters Associates (Milford, MA) 6000A pump, U6K septumless injector, Schoeffel variable wavelength UV detector (Westwood, NJ), and Omni-Scribe recorder (Houston Instrument, Austin, TX). Operating conditions: injection volume, 10 μL; flow rate, 2.5 mL/min; wavelength, 280 nm; absorbance range, 0.4 AUFS; recorder setting, 10 mV; chart speed, 1.0 cm/min.

(b) *Chromatographic column.*—10 cm × 8 mm id polyethylene column containing μBondapak C_{18} Radial-Pak (Waters Associates).

(c) *Mobile phase.*—Acetonitrile-methanol-tetrahydrofuran-water (34 + 30 + 1 + 35). All solvents HPLC grade (Fisher Scientific, Fair Lawn, NJ).

(d) *Sample extraction solvent.*—Reagent grade dioxane (Fisher Scientific).

(e) *Rotenone standard solution.*—Weigh 20 mg 99% rotenone (Aldrich Chemical Co., Inc., Milwaukee, WI) into actinic 50 mL volumetric flask and dilute to volume with reagent grade dioxane (actinic glassware is necessary because rotenone degrades readily in most types of light).

Preparation of Sample

(a) *Dust.*—Weigh sample equivalent to 20 mg rotenone into glass-stopper Erlenmeyer flask. Pipet 50 mL dioxane and sonicate 5 min while swirling gently. Let settle and filter 5–10 mL aliquot through 0.45 μm filter (Millipore Corp., Bedford, MA). Inject 10 μL into HPLC system.

(b) *Liquid.*—Weigh sample equivalent to 20 mg rotenone into glass-stopper Erlenmeyer flask. Pipet 50 mL dioxane and shake to dissolve liquid. Filter through 0.45 μm filter (Millipore Corp.). Inject 10 μL into HPLC system.

Determination

Inject standard followed by 2 injections of sample and finally inject another standard. Measure peak heights, average, and substitute into formula below:

$$\% \text{ Rotenone} = (H/H') \times (W'/W) \times \% \text{ purity of std}$$

where H and H' = average peak heights of sample and standard, respectively; W' = g rotenone standard/50 mL; and W = g sample extracted.

Results and Discussion

Five commercial formulations (a representative sampling of rotenone formulations) were analyzed by radial compression HPLC. The

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R. J. Bushway is the Associate Referee on Rotenone and Other Rotenoids.

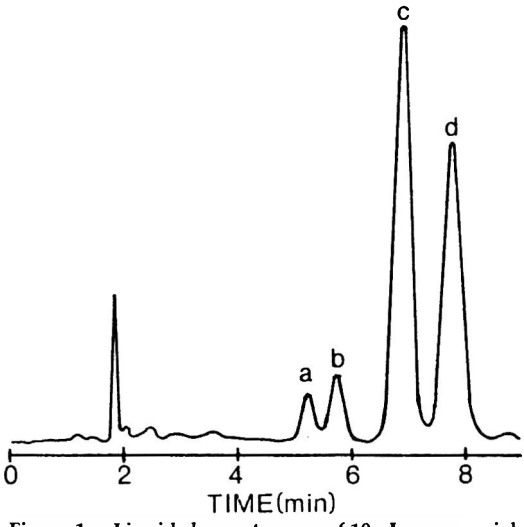

Figure 1. Liquid chromatogram of 10 μL commercial sample: a, rotenolone; b, tephrosin; c, rotenone; d, deguelin. See Method for operating conditions.

formulations varied in the amount of rotenone, other rotenoids, and pesticides. Sample 1 was a liquid; the others were dusts. Amount of rotenone guaranteed varied from a low of 0.75% to a high of 20%, while the quantity of other rotenoids present ranged from 0.75% to 10%. Sample 5 was also formulated with 4.7% captan, 0.3% of other captan derivatives, and 5% methoxychlor.

A typical chromatogram of an HPLC separation of a rotenone formulation is shown in Figure 1. Rotenone eluted in approximately 7 min and was preceded by retenolone and tephrosin, while deguelin was the last major rotenoid to elute from the column. These 3 rotenoids are the ones normally formulated with rotenone, but several others could be present. Thus, any method for rotenone analysis must be able to separate rotenone from other rotenoids. Table 1 gives the relative retention times of 11 rotenoids that could be present in rotenone formulations. None of these rotenoids was observed to interfere with rotenone analysis, but if dihydrodeguelin was present at high levels, it could interfere with the determination of rotenone. However, dihydrodeguelin would not be present at high concentrations in rotenone formulations.

Rotenone is sometimes formulated with other pesticides such as carbaryl (1-naphthol may be present as the breakdown product), folpet, captan, difolatan, methoxychlor, piperonyl butoxide, and pyrethrins. Retention times relative to rotenone of these pesticides are shown in Table 1. Like the rotenoids, none of these pesticides interfered with rotenone analysis.

A recovery study was not performed because in a previous method (2) it had been demonstrated that dioxane with $1\frac{1}{2}$ h on a rotary shaker extracted rotenone with a recovery of 96%. However, the new ultrasonication procedure was compared with the rotary shaker method. All samples were extracted in duplicate so each formulation could be analyzed using both procedures. Each extraction method (within experimental error) gave the same percent rotenone values, indicating that the ultrasonication method was a good way to extract rotenone formulations.

In a previous method (2), the linearity of rotenone in a reverse phase system had been shown. In rechecking this linearity, it was observed that the concentration of rotenone could be calculated using the peak height by direct comparison with the standard.

Table 1. Relative retention times for rotenoids and pesticides and their degradation products that could be present in rotenone formulations

Rotenoid	RR	Pesticide or product	RR
L-Elliptone	0.61	Carbaryl	0.42
Rotenolone	0.75	1-Naphthol	0.49
Tephrosin	0.82	Captan	0.63
Rotenone	1.00	Folpet	0.83
Dihydrodeguelin	1.09	Difolatan	0.89
Deguelin	1.12	Rotenone	1.00
Sumatrol	1.43	Methoxychlor	0.58, 0.60, 1.46, 1.57, 2.42[a]
Toxicarol	1.67	Piperonyl butoxide	0.57, 0.68, 0.81, 1.25, 1.36, 1.53, 1.69, 1.90, 2.42[a]
Isorotenone	1.78	Pyrethrins	1.81, 1.98, 2.47, 2.67[a]
Dehydrorotenone	2.07		
Rotenonone	2.39		
Dehydrodeguelin	2.49		

[a] Principal peak.

Table 2. Analysis of rotenone formulations

Sample	Formulated, %	Found,[a] %	CV, %
1 Liquid	5.00	4.84	1.32
2 Dust	20.00	18.37	1.58
3 Dust	5.00	4.86	1.63
4 Dust	1.00	0.99	0.91
5 Dust	0.75	0.89	1.46

[a] Means of 11 different determinations for each sample.

To demonstrate the reproducibility of this HPLC method, 5 commercial formulations were analyzed 11 times each for their rotenone content. The analyses were performed over a period of 6 days with 2 analyses of each performed the first 5 days and 1 on the last day. The results are given in Table 2. Means for 3 of the samples were close to the guarantee values while one was over-formulated and one was under-formulated. The percent coefficients of variation were excellent with all below 1.65%, indicating that the method from day-to-day is very reproducible.

The linearity of rotenone is such at 280 nm that higher or lower concentrations could be measured using this method. Also for low levels of rotenone, this method has the sensitivity required for residue methods (8 ng could be detected using an attenuation of 0.02 AUFS), provided there are no interferences.

In conclusion, this HPLC method for the analysis of rotenone formulations is simple, rapid, and precise. Sample extraction and elution time of rotenone is much shorter than with the previous methods (1, 2). Radial compression offers rapid high performance liquid chromatography.

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High Performance Liquid Chromatographic Analysis of Rotenone Formulations: Collaborative Study

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A collaborative study was conducted on a high performance liquid chromatographic (HPLC) method for determining rotenone in formulations. One liquid and 4 dust samples, containing other rotenoids and pesticides, with rotenone guarantees ranging from 0.75% to 20% were analyzed by 16 laboratories. Peak height measurements were used by 14 laboratories while 8 laboratories used integrator area measurements. Samples were extracted on a rotary shaker for 1½ h with dioxane. An aliquot was analyzed using reverse phase HPLC and UV detection at 280 nm. Coefficients of variation ranged from 2.47 to 3.19% for peak height determinations and from 2.56 to 6.00% for peak area determinations. One collaborator analyzed the samples by the official infrared method for a comparison. The HPLC method has been adopted official first action.

Rotenone is a naturally occurring insecticide found in many leguminous species of the genera *Derris*, *Lonchocarpus*, *Amorpha*, and *Tephrosia* (1). It is formulated with a variety of other rotenoids and pesticides, which makes the analysis of rotenone in formulations very complex.

Of the methods available for the determination of rotenone in formulations (1), high performance liquid chromatography is preferred because it is rapid, precise, and accurate and can separate rotenone from other rotenoids and pesticides. Analysis of rotenone formulations by HPLC was first performed using normal phase (silica) chromatography (2). Because of the instability of silica columns, a reverse phase separation was developed in 1977 for rotenone formulations (3). A modification of this method (3) was subjected to a collaborative study which is reported herein.

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The recommendation of the Associate Referee was accepted 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.* (1983) **66**, 333; 412.

Collaborative Study

Collaborators were furnished with 5 samples (Table 1) varying in rotenone concentration, other rotenoids, and pesticides and were asked to analyze each in duplicate. Also supplied were a method, special instructions as to the injection format, a sample chromatogram, rotenone analytical standard, and report forms. Before beginning the study, each collaborator was asked to check the column performance by analyzing Sample 1, which was the most difficult of the 5 to chromatograph accurately, and to adjust the mobile phase if needed. This separation was to be compared with the sample chromatogram included. Collaborators were requested to use peak height measurements in determining the percent rotenone and if possible to use peak area by electronic integration, too. Finally it was asked that all data and chromatograms be returned.

Rotenone in Formulations
Liquid Chromatographic Method
Official First Action

6.D05 ***Principle***

Sample is extd with dioxane, and rotenone is detd by reverse phase HPLC with UV detection at 280 nm.

6.D06 ***Apparatus and Reagents***

(**a**) *Liquid chromatograph.*—M6000A pump, U6K injector, Model 450 variable UV detector (all Waters Associates, Inc.), and Omni-Scribe recorder (Houston Instrument, Austin, TX 78753), or equiv. system. Operating conditions: column ambient; flow rate 1.0 mL/min for Partisil column, 1.5 mL/min for Zorbax column, 1.2 mL/min for Bondapak column; injection vol. 5

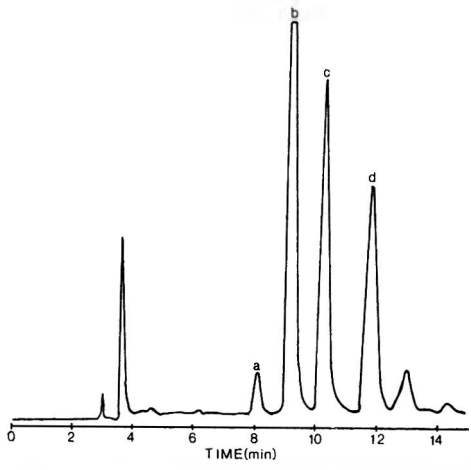

Figure 6:D1. Liq. chromatogram of rotenone sample with Whatman column: a, rotenolone; b, tephrosin and an unknown; c, rotenone; d, deguelin.

μL for Partisil column and 10 μL for others; detector wavelength 280 nm; absorbance range 0.4 AUFS; chart speed 1 cm/min.

(b) *Chromatographic columns.*—Partisil 5 ODS-3, 5 μm particle size, stainless steel, 25 cm × 4.6 mm id (Whatman Inc., Clifton, NJ 07014). Zorbax C_8, 10 μm particle size, stainless steel, 25 cm × 4.6 mm id (DuPont Co.). μBondapak C_{18}, 10 μm particle size, stainless steel, 30 cm × 3.9 mm id (Waters Associates, Inc.).

(c) *Mobile phases.*—Use LC grade org. solvs (Fisher Scientific Co.). Use glass-distd H_2O treated to remove org. compds by passing thru C_{18} column system (Millipore Corp., Bedford, MA 01730), or use HPLC grade H_2O. Use MeOH–H_2O (75 + 25), (68 + 32), and (66 + 34) for Partisil, Zorbax, and Bondapak, resp. If necessary, adjust mobile phase to give adequate sepn of tephrosin, rotenone, and deguelin in test soln (Fig. 6:D1).

(d) *Test soln.*—Accurately weigh portion of well mixed sample of Noxfish Fish Toxicant or powd cubé root ext (Penick Co., Lyndhurst, NJ 07071) contg ca 20 mg rotenone into 125 mL g-s erlenmeyer. Add 50 mL dioxane, and mix.

(e) *Std soln.*—Accurately weigh ca 20 mg 99% pure rotenone (Penick Co.) into 50 mL vol. flask and dil. to vol. with dioxane (reagent grade). Keep rotenone from light or store in actinic glassware.

(f) *Sample extraction solvent.*—Reagent grade dioxane.

6.D07 *Preparation of Sample*

(a) *Solid formulations.*—Accurately weigh portion of well mixed sample contg ca 20 mg rotenone into 125 mL g-s erlenmeyer. Pipet in 50 mL dioxane, stopper, and shake 1½ h on rotary shaker. Let settle and filter aliquot thru 0.45 μm organic filter (Millipore Corp.), or equiv.

(b) *Liquid formulations.*—Use same procedure as above, omitting rotary shaking and settling.

Table 1. Composition of 5 commercial formulations sent to collaborators

Sample	Rotenone, %	Other rotenoids, %	Other pesticides, %
1 Liquid	5.0	10.0	0
2 Dust	1.0	0.05	0
3 Dust	5.0	0.10	0
4 Dust	20.0	5.0	0
5 Dust	0.75	0.75	4.7,[a] 0.3,[b] 5.0[c]

[a] Captan.
[b] Related derivatives of captan.
[c] Methoxychlor.

6.D08 *Determination and Calculation*

Inject std soln followed by 2 injections of sample soln and another injection of std soln. Measure peak hts, average, and calc. as follows:

$$\% \text{ Rotenone} = (PH/PH') \times (W'/W) \times \% \text{ purity of std}$$

where PH and PH' = av. peak hts of sample and std solns, resp.; W' = g rotenone std/50 mL; and W = g sample extd.

Results and Discussion

Because rotenone formulations contain other rotenoids and often other pesticides, rotenone analysis is complex. Tephrosin, rotenolone, and deguelin are the rotenoids most prevalent in rotenone formulations (Figure 6:D1). To obtain baseline separation of rotenone from tephrosin and deguelin, a column in good condition with many theoretical plates is required. Other rotenoids such as dehydrorotenone, rotenonone, and dehydrodeguelin, which also may be present, eluted after deguelin, and in some formulations may occur in sufficient quantities to cause problems as late-eluting peaks. The pesticides that are sometimes formulated with rotenone are carbaryl, folpet, difolatan, pyrethrins, piperonyl butoxide, and methoxychlor. As with the rotenoids, none of these compounds interferes with rotenone analysis by HPLC although

caution must be used because compounds like piperonyl butoxide, methoxychlor, and pyrethrins have late-eluting peaks which can interfere with subsequent injections.

Samples for this study were chosen to give a representative sampling of rotenone formulations (Table 1). These formulations varied in the amount of rotenone, other rotenoids, and pesticides. All samples were obtained from commercial manufacturers of rotenone formulations.

Table 2 presents the results of the collaborative study based on peak heights; integrated peak area results are shown in Table 3. Only 3 outliers were observed at the 98% confidence limit for peak height determinations. Coefficients of variation ranged from 2.47 to 3.19% which are somewhat higher than many collaborative formulations methods. But considering that there was no internal standard and the complexity of the separations was such that it was virtually impossible to have an internal standard, the results were good.

Precision results from peak area calculations were not as good as peak height calculations (Tables 2 and 3), although the means for both were almost identical. Coefficients of variation ranged from 2.56 to 6.00%. This greater variation was due to the fact that not all collaborators obtained complete separation between tephrosin and rotenone. Thus, the variation was caused by the different ways that the collaborators programmed their integrators to measure areas in this situation. The problem was not encountered with peak height analysis because everything was measured the same way, from baseline to baseline. Furthermore, 2 laboratories reported peak area data so discrepant from their peak height data that the area data were disregarded. Apparently this was because of malfunctioning integrators. From these results (Tables 2 and 3), the Associate Referee would recommend using peak height measurements (baseline to baseline) for rotenone concentration if tephrosin is not resolved from rotenone. However, if both compounds are separated, then either area or peak height can be used. The data obtained from the collaborators where tephrosin was resolved from rotenone support this statement.

Because of the complex nature of this analysis, there were several comments from collaborators. For the most part, collaborators found the method straightforward with only minor problems. Collaborator 2 had to alter the mobile phase for the Whatman column from 75 + 25 (methanol–water) to 70 + 30 to obtain baseline separation of rotenone. This is a common occurrence in HPLC analyses because of the way columns are manufactured or because of the age of the column. Collaborator 6 had to purchase a new column after analyzing Sample 1 because of efficiency loss. This was not encountered by other collaborators so the column must have been old or defective. Also, Collaborator 6 did not have a gyratory shaker so he used a magnetic stirrer which seemed to work well. Collaborators 9 and 12 observed 2 peaks after injecting the rotenone standard. Both were injecting 20 μL on a μBondapak C_{18} column. As reported in an earlier paper (3), certain volumes of dioxane on such a column cause problems. Collaborator 9 decreased the injection volume after conferring with the author, while Collaborator 12 used methanol as the extraction and standard solution solvent. This change did not affect the results. Collaborator 7 noticed a column temperature increase from 27.5 to 32.1°C along with a steady increase in peak height of the standard during the analysis. Collaborator 6 observed an increase in peak height for Sample 5 only. This does not appear to be a major problem, but if a consistent increase in peak height is encountered during the analysis, a temperature control system may be appropriate. Collaborator 13 had difficulty with Regis and Varian columns although Collaborators 4 and 8 used these columns without problems. Again, the columns may have been old or defective. Collaborator 14 observed the standard degrading over a period of a week. Extreme care must be used to keep rotenone from light, so actinic glassware should be used. Also, one should make sure the dioxane is peroxide-free because peroxides can also degrade rotenone.

Some collaborators made suggestions to improve the method. Collaborators 2, 7, and 9 recommended that the extracts be allowed to settle after extraction and be directly filtered through the 0.45 μm filters instead of filtering through Whatman 2v paper followed by the 0.45 μm filter. This suggestion was adopted. Collaborator 2 also recommended the use of acetonitrile instead of methanol to obtain lower back pressures with 5 μm packing. This must be thoroughly examined before a change can be made because of possible interferences. However, if it works, it should lengthen column life. Collaborator 7 suggested that the dioxane should be freshly distilled or checked for peroxides. Collaborator 9 as mentioned previously reported using methanol in place of dioxane for sample

Table 2. Collaborative results for rotenone (%) by peak height measurements

Coll.	1A	1B	Diff. (1A − 1B)	2A	2B	Diff. (2A − 2B)	3A	3B	Diff. (3A − 3B)	4A	4B	Diff. (4A − 4B)	5A	5B	Diff. (5A − 5B)
1	4.72	4.60	+0.12	0.94	0.94	0	4.65	4.60	+0.05	17.82	18.20	−0.38	0.86	0.85	+0.01
2	4.96	4.90	+0.06	1.01	1.04	−0.03	4.97	5.04	−0.07	18.54	18.78	−0.24	0.88	0.90	−0.02
3	4.73	4.67	+0.06	0.97	1.00	−0.03	4.91	4.81	+0.10	18.14	17.87	+0.27	0.91	0.91	0
4	4.73	4.73	0	0.99	1.00	−0.01	4.75	4.73	+0.02	17.80	17.80	0	0.90	0.88	+0.02
5	4.64[a]	5.89[a]	—	1.01	0.99	+0.02	4.96	4.96	0	18.65	18.33	+0.32	0.88	0.86	+0.02
6	4.87	4.88	−0.01	0.98	0.98	0	4.89	4.80	+0.09	17.67	17.73	−0.06	0.88	0.91	−0.03
7	4.57	4.71	−0.14	0.95	0.99	−0.04	4.83	4.75	+0.08	18.00	17.90	+0.10	0.83	0.85	−0.02
8	4.89	4.92	−0.03	1.05	0.98	+0.07	4.94	5.06	−0.12	18.32	17.55	+0.77	0.86	0.90	−0.04
9	4.56	4.60	−0.04	0.99	0.99	0	4.85	4.80	+0.05	18.22	18.90	−0.68	0.88	0.88	0
10	4.87	4.85	+0.02	0.95	0.97	−0.02	4.90	4.78	+0.12	18.37	18.22	+0.15	0.93	0.90	+0.03
11	4.83	4.82	+0.01	1.01	1.00	+0.01	4.95	5.15	−0.20	18.69	18.84	−0.15	0.95	0.92	+0.03
12	4.72	4.70	+0.02	0.99	0.99	0	4.86	4.87	−0.01	18.80	18.58	+0.22	0.93	0.93	0
13	4.73	4.65	+0.08	0.94	0.91	+0.03	4.42[a]	4.52[a]	—	17.10	18.10	−1.00	0.89	0.91	−0.02
14	4.64[a]	4.91[a]	—	0.99	1.03	−0.04	4.82	4.92	−0.10	17.39	17.62	−0.23	0.91	0.91	0
N		24			28			26			28			28	
Mean		4.76			0.985			4.87			18.14			0.893	
S_r		0.046			0.020			0.066			0.304			0.015	
S_b		0.108			0.024			0.104			0.365			0.024	
S_d		0.118			0.031			0.124			0.476			0.028	
CV, %		2.47			3.19			2.54			2.62			3.19	

[a] Outlier laboratory results omitted from calculations. Determined at the 98% confidence limit.

Table 3. Collaborative results for rotenone (%) by integrator area

Coll.	1A	1B	Diff. (1A − 1B)	2A	2B	Diff. (2A − 2B)	3A	3B	Diff. (3A − 3B)	4A	4B	Diff. (4A − 4B)	5A	5B	Diff. (5A − 5B)
3	4.86	4.89	−0.03	0.99	1.00	−0.01	4.97	4.86	+0.11	18.22	18.33	−0.11	0.91	0.93	−0.02
4	4.80	4.73	+0.07	0.99	0.98	+0.01	4.72	4.66	+0.06	18.00	17.60	+0.40	0.90	0.86	+0.04
6	4.89	4.88	+0.01	0.99	0.97	+0.02	4.91	4.75	+0.16	17.73	17.88	−0.15	0.88	0.89	−0.01
7	5.05	4.86	+0.19	0.95	0.97	−0.02	4.70	4.66	+0.04	17.98	17.81	+0.17	0.82	0.86	−0.04
8	5.35	5.39	−0.04	1.02	1.00	+0.02	4.97	5.03	−0.06	17.83	17.55	+0.28	1.03	0.97	+0.06
9	4.76	4.81	−0.05	1.00	1.00	0	4.78	5.06	−0.28	18.33	19.13	−0.80	0.87	0.92	−0.05
11	4.70	4.70	0	1.00	0.98	+0.02	4.90	5.10	−0.20	18.50	18.70	−0.20	0.98	0.95	+0.03
15	4.53	4.56	−0.03	0.95	0.92	+0.03	4.64	4.67	−0.03	17.21	17.07	+0.14	0.88	0.86	+0.02
N		16			16			16			16			16	
Mean		4.86			0.982			4.84			17.99			0.907	
S_r		0.054			0.013			0.102			0.250			0.026	
S_b		0.231			0.022			0.121			0.472			0.048	
S_d		0.237			0.025			0.158			0.535			0.054	
CV, %		4.88			2.56			3.26			2.97			6.00	

extraction. The results were good with methanol. It appears that methanol could replace the more toxic dioxane. Furthermore, the author has tried a 5 min dioxane ultrasonication of these samples in place of the 1½ h shaking technique and the results were comparable to the 1½ h method. Thus, the 5 min procedure is recommended. This new extraction has not been tried with methanol. Collaborators 6 and 7 reported that a 30 to 45 min interval should be used between sample injections because of late-eluting peaks; such was the case with samples 1 and 5. This recommendation should be followed. Finally, Collaborator 6 suggested that a significant improvement might be the addition of reproducibility requirements for sample and standard injections; this was adopted.

Ideally, a collaborative study should be organized such that all collaborators use the same conditions. However, this is difficult with HPLC collaborative methods because of the expense and wide variety of instrumentation and columns. With this in mind, this collaborative study was designed to give collaborators a choice of 3 columns and 3 solvent systems. Even with these choices, there were 3 other columns used in this study. There were also 6 different pump and 7 different injection systems. The injectors included variable loops, fixed loops, and auto samplers. Furthermore, Collaborator 12 used a different extraction procedure, but obtained comparable results. None of the changes are consistent among the outliers. Thus, these variations are just added proof of the ruggedness of this method.

Collaborator 16 analyzed the samples by the official IR method. The results were as follows: 1, 3.35%; 2, 0.92%; 3, 4.35%; 4, 16.7%; 5, 3.18%. Compared to the results obtained from the HPLC method, the first 4 samples were on the low side while 5 was on the high side. Sample 5 was high because of interferences from the other pesticides. For samples containing just rotenone and the other rotenoids, it appears that the IR method gives results with a low bias and samples containing other pesticides may contain interferences for the IR method.

Recommendation

Because of the complexity of rotenone analysis in formulations, the alternative methods available, the variety of chromatographic conditions used in this study, and the good results obtained, it is recommended that the HPLC method be adopted official first action.

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High Pressure Liquid Chromatographic Determination of Fensulfothion: Collaborative Study

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A method for analyzing fensulfothion was tested by 10 collaborators. Formulations were dissolved, or extracted from inerts, in methanol. Benzophenone was used as an internal standard. The solution was chromatographed on a Partisil-10 ODS-2, or equivalent, reverse phase column, and detected at 230 nm. A mobile phase of methanol-water-phosphoric acid was used. The ratio of fensulfothion peak height to benzophenone peak height was calculated from the UV response and compared to the standard material for quantitation. A 15% granular formulation was analyzed as a matched pair. The results of one collaborator were outliers by the Dixon test. The coefficient of variation for the granular formulation was 1.6%. A matched pair of 63% spray concentrate samples was analyzed by 10 collaborators. The difference in results was an outlier for one collaborator; the coefficient of variation for the other collaborators was 1.5%. The method has been adopted official first action.

Fensulfothion (Dasanit; *O,O*-diethyl *O*-[4-(methylsulfinyl)phenyl] phosphorothioate) is a compound exhibiting both insecticidal and nematicidal activity. Fensulfothion kills both insects and nematodes primarily by contact action.

Previous methods for the determination of fensulfothion were nonspecific. Our laboratory has successfully used high pressure liquid chromatography for analyzing fensulfothion formulations for about 3 years, and has found that it is a fast, precise method.

Collaborative Study

Ten collaborators were sent 2 samples of 15% granular fensulfothion as a matched pair, and 2 samples of 63% spray concentrate also as a matched pair. In addition to the samples, collaborators received an analytical standard, an internal standard, and a copy of the method. Each collaborator was asked to make duplicate injections of each sample followed by a standard injection. They were then to calculate average ratios of fensulfothion peak heights to internal standard peak heights for each set of duplicate injections and to calculate % fensulfothion.

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The recommendation of the Associate Referee was accepted by the General Referee and Committee A and was adopted by the Association. See the Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, 335; 411.

Fensulfothion (*O,O*-Diethyl *O*-[*p*-(Methylsulfinyl)phenyl] Phosphorothioate) in Formulations

Liquid Chromatographic Method

Official First Action

6.D28 ***Principle***

Sample is dissolved in or extd with MeOH, benzophenone is added as internal std, and fensulfothion is detd by liq. chromatgy and UV detection at 230 nm.

6.D29 ***Apparatus***

(a) *Liquid chromatograph.*—Able to generate >1000 psi and equipped with detector able to measure *A* at 230 nm. Typical operating conditions: temp., ambient; flow rate, 0.8 mL/min; wavelength, 230 nm; chart speed, 2 mm/min; sample size, 10 μL. Conditions may be varied to accommodate instrument and column differences.

(b) *Column.*—Whatman Partisil PXS 10/25 ODS-2, stainless steel 25 cm × 4.6 mm id (Whatman, Inc., Clifton, NJ 07014), or equiv.

(c) *Filter.*—10 μm Teflon, or similar type.

6.D30 ***Reagents***

(a) *Methanol.*—Distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).

(b) *Phosphoric acid.*—85% (Fisher Scientific Co.).

(c) *Internal std soln.*—0.25 mg benzophenone/mL. Accurately weigh ca 250 mg benzophenone (Eastman Kodak Co.) into small flask. Transfer to 1 L vol. flask and dil. to vol. with same MeOH to be used in mobile phase. Concn may

be varied so that when std soln (**d**) is injected, peak ht of benzophenone matches peak ht of fensulfothion within 20%.

(**d**) *Std soln.*—0.3 mg fensulfothion/mL, within optimum linearity range. Accurately weigh ca 150 mg fensulfothion (Mobay Chemical Co., Box 4913, Kansas City, MO 64120) into 125 mL flask. Pipet in 100 mL MeOH, shake to mix. Pipet 10 mL aliquot of soln into 125 mL flask with screw cap, add exactly 40 mL internal std soln, and shake to mix. Prep. fresh std daily. Keep ref. std in freezer.

(**e**) *Mobile phase.*—MeOH-H_2O (80 + 20) buffered to 0.0025M with H_3PO_4. Mix 800 mL MeOH + 200 mL H_2O + 156 μL H_3PO_4, and degas. If using column other than ODS-2, adjust MeOH-H_2O ratio as necessary.

6.D31 *Preparation of Sample*

(**a**) *Spray concentrate.*—Accurately weigh sample contg ca 150 mg fensulfothion into 125 mL flask. Pipet in 100 mL MeOH and shake to mix. Pipet 10 mL aliquot into 125 mL flask with screw cap, add exactly 40 mL internal std soln, and shake to mix.

(**b**) *Granular formulations.*—Pour sample into 400 mL beaker and thoroly mix, turning granules over ≥10 strokes with wide spatula. Take weighed amt from beaker before sample is poured back into sample container. Accurately weigh sample contg ca 150 mg fensulfothion into 125 mL flask. Pipet in 100 mL MeOH and place on mech. shaker 15-30 min. Filter thru 10 μm Teflon or similar type filter. Place 10 mL aliquot of filtrate in 125 mL flask with screw cap, add exactly 40 mL internal std soln, and shake to mix.

6.D32 *Determination*

Adjust liq. chromatgc operating parameters to elute fensulfothion in 4-7 min. Maintain all parameters const thruout analysis. Benzophenone will elute 2-4 min after fensulfothion.

Adjust injection size and attenuation to give 60-80% on-scale peaks. Make repetitive injections of std until response is stable, and ratios of fensulfothion peak ht to benzophenone peak ht for successive injections vary ≤1%. Then make duplicate injections of sample followed by injection of std. Calc. av. ratio of fensulfothion peak ht to benzophenone peak ht for each set of duplicate injections and calc. % fensulfothion.

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

where R and R' = av. peak ht ratios for sample and std, resp.; W = mg sample; W' = mg fensulfothion anal. std; and P = % purity of fensulfothion anal. std. Integrator area ratios may be substituted for peak ht ratios.

Table 1. Collaborative results for fensulfothion in 15% granular samples

Coll.	Sample 1	Sample 2	Diff.	Total
1	14.75	14.80	−0.05	29.55
2	14.57	14.70	−0.13	29.27
3	15.28	14.71	0.57	29.99
4	14.95	14.54	0.41	29.49
5	14.97	15.05	−0.08	30.02
6	14.74	14.97	−0.23	29.71
7	14.86	14.95	−0.09	29.81
8	15.35	15.24	0.11	30.59
9	15.02	14.97	0.05	29.99
10[a]	13.59	13.57	—	—
Av.	14.94	14.88	0.062	29.82
S_d				0.27
S_r			0.19	
S_b			0.14	
$\sqrt{S_r^2 + S_b^2}$			0.23	
CV, %			1.57	

[a] Results are outliers by Dixon test. Not included in statistical evaluation.

Results and Discussion

Results were received from all 10 collaborators. Comments from various collaborators indicated that they found that the method was quick and simple and gave good results.

Table 1 shows results for the 15% granular samples. Results of Collaborator 10 were outliers by the Dixon test, and were omitted from the statistical evaluation. The coefficient of variation for the remaining data was 1.57%.

Table 2 shows results for the 63% spray concentrate samples. The difference in results for one collaborator was an outlier. Evaluation excluding that data resulted in a coefficient of variation of 1.47%.

One collaborator questioned the fensulfothion peak not being adequately separated from impurities and suggested a longer run time. The increased time could be easily accomplished by varying the ratio of methanol and water. Although the Associate Referee tried different wavelengths, 230 nm seemed to work very well in our laboratory. There should be no problem using 254 nm as was suggested by 1 or 2 collaborators.

Of 10 collaborators, 3 used the ODS-2 column, 2 used a Bondapak C_{18} column, and others used various reverse phase columns such as Micropak MCH-18, and Alltech C_{18}. There were no no-

Table 2. Collaborative results for fensulfothion in 63% spray concentrate samples

Coll.	Sample 3	Sample 4	Diff.	Total
1	60.99	60.27	0.72	121.26
2	63.67	62.01	1.66	125.68
3	62.28	60.99	1.29	123.27
4	62.18	62.41	−0.23	124.59
5	61.54	61.42	0.12	122.96
6	60.06	59.91	0.15	119.97
7	61.17	61.49	−0.32	122.66
8	62.13	61.51	0.62	123.64
9[a]	59.09	62.21	−3.12	121.30
10	61.47	61.03	0.44	122.50
Av.	61.72	61.23	0.133	122.78
S_d				1.19
S_r			0.47	
S_b			0.78	
$\sqrt{S_r^2 + S_b^2}$			0.91	
CV, %			1.47	

[a] The difference for this laboratory is an outlier. Not included in statistical evaluation.

ticeable differences due to the use of these various columns.

Recommendation

The Associate Referee recommends that the HPLC method for fensulfothion be adopted official first action.

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High Pressure Liquid Chromatographic Determination of Impurity Phenols in Technical 2,4-D Acid and 2,4-Dichlorophenol

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Thirteen technical 2,4-dichlorophenoxyacetic (2,4-D) acid and five 2,4-dichlorophenol (2,4-DCP) samples were obtained from several manufacturers and analyzed for the presence of various chlorinated phenolic impurities. Reverse phase high pressure liquid chromatography with a UV detector in series with an electrochemical detector was used for qualitative and quantitative determinations. Three solvent systems were investigated in the present study. The retention times obtained for various mono-, di-, and tri-chlorophenols with each solvent system are presented. The presence of *ortho*- and *para*-monochlorophenol (averages 1.23 and 0.41%, respectively), 2,6-dichlorophenol (average 4.5%), 2,4,6-trichlorophenol, and a dichloro-methyl phenol isomer were detected as contaminants in technical 2,4-DCP samples. Technical 2,4-D acid contained the same type of chlorophenol impurities but in much smaller amounts. A comparison of the sensitivity of a UV detector at 280 nm with an electrochemical detector showed the latter to be 5 to 15 times more sensitive, depending on the chlorophenol.

In recent years the role of chemical impurities in pesticide technical materials and formulations has greatly increased in importance. This has had an impact on the analytical techniques used in their detection and quantitation. In the case of the low levels of neutral impurities, such as chlorinated dioxins, in 2,4-D and 2,4,5-T the approach has been via high resolution gas chromatography/mass spectrometric procedures (1, 2) while high pressure liquid chromatography (HPLC) is used for the higher levels of acidic and phenolic impurities that may be present (3, 4). The identity of the various chlorophenols is especially important because condensation (e.g., 2,4,5- and 2,4,6-trichlorophenols) during their manufacture under alkaline conditions can yield chlorinated dioxins which can be carried through into the final products, e.g., 2,4,5-T (5). Since 2,4-dichlorophenol (2,4-DCP) is produced by the chlorination of phenol under acidic conditions, low temperatures, and pressure, it was postulated that the formation of the expected 2,7-dichloro-dibenzodioxin isomer would be unlikely (5). However, the manufacture of 2,4-D acid involves the reaction of 2,4-DCP with chloroacetic acid under basic aqueous conditions (6) which may favor the possible formation of small amounts of toxic dioxins by self- and cross-condensation of the various chlorophenols that may be present. To date, a quantitative chlorophenol impurity analysis of 2,4-DCP and 2,4-D acid has not been reported, although an isomer-specific (3) and other (7) HPLC methods are available.

The present investigation assayed a number of technical 2,4-DCPs and simultaneously compared the utility of multi-wavelength UV absorbance and an electrochemical detector for this type of analysis.

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Experimental

Apparatus

(a) *High pressure liquid chromatograph.*—Waters Associates Model 6000A solvent delivery system equipped with Waters U6K universal injector and Schoeffel Instrument Model SF 770 Spectroflow multiwavelength detector, set at 208 or 280 nm, sensitivity 0.01 AUFS connected in series with electrochemical detector LCEC (Bioanalytical Systems) with a glassy carbon electrode (TL-5) and a reference electrode (RE1) set at positive voltage of 0.85 V, sensitivity 0.2, and 0.5 nanoamp/V × 100B (B = 2 s), background current offset − 10^3 nA.

(b) *Recorders.*—Westronics, Inc., Model MT linear recorder 115 V, span 10 mV/in. (with UV detector) and 1 V/in. (with electrochemical detector), chart speed 15 in./h.

(c) *HPLC column.*—25 cm × 4.6 mm id packed with μLiChrosorb RP-18 10 μm material (Brownlee Labs, Santa Clara, CA) connected to a 7.6 cm × 2 mm Co:Pell ODS guard column (Whatman Inc., Clifton, NJ).

Solvents and Reagents

(a) *Buffer solutions.*—0.3M, 0.03M, and 0.09M solutions of NaH_2PO_4 in deionized water.

(b) *Mobile phases.*—(i) Acetonitrile–0.3M buffer (25 + 75) adjusted to pH 4.4 with H_3PO_4. (ii) Acetonitrile–0.03M buffer (35 + 65) pH ad-

Table 1. Retention time (min) for 2,4-D acid and related compounds on LiChrosorb RP-18 reverse phase column, using different solvent systems

Solvent system	Compound	Buffer 0.03M	Buffer 0.09M	Buffer 0.3M
1. 35% CH_3CN–buffer, pH 5.45, 2.7 mL/min	2,4-D acid	0	0	
	2-chlorophenol	4.95	5.20	
	4-chlorophenol	5.85	6.60	
	3-chlorophenol	6.30	—	
	2,6-DCP	8.48	9.2	
	2,4-DCP	11.32	11.18	
	2,5-DCP	11.41	—	
	2,3,6-TCP	16.42	—	
	2,3,4-TCP	19.27	—	
	2,4,6-TCP	20.40	21.65	
	2,4,5-TCP	23.32	23.23	
	2,4-dichloro-6-methyl phenol	23.32	23.3	
	2,3,5-TCP	24.75	—	
2. 35% CH_3CN–buffer, pH 4.4, 2 mL/min	2,6-DCP			11.18
	2,4-DCP			13.20
	2,4,6-TCP			26.5
	2,4,5-TCP			27.06
3. 25% CH_3CN–buffer, pH 4.4, 2 mL/min	2,4-D			9.07
	2,6-DCP			15.89
	2,4-DCP			27.52

justed to 5.45. Pass solutions through 0.45 μm filter.

(c) *Potassium hydroxide solution.*—0.2M KOH in 2-propanol–water (1 + 1).

(d) *Standard stock solution.*—Accurately weigh ca 50 mg of each pure chlorophenol (standard) (see Table 1) and dilute to 50 mL with KOH solution.

(e) *Analytical solutions.*—Dilute 100 μL of each stock solution to 10 mL with KOH solution to give final concentrations of 0.2, 0.5, 1, 2, 5, and 10 ng/μL, respectively.

(f) *Technical materials.*—Dissolve 600 mg 2,4-D or 2,4-DCP sample in 50 mL KOH solution and further dilute 1 mL of this stock solution to 100 mL.

(g) *Solvents.*—Acetonitrile (HPLC grade, Caledon Labs Ltd, Georgetown, Ontario). Water (purified with a Mille-Q2 system and filtered through 0.45 μm filter). 2-Propanol (Fisher Scientific Co.).

Determination

Step 1: Inject 5 μL aliquots of diluted technical material solutions until reproducible retention times and peak areas are obtained (variation ≤2%).

Step 2: Proceed with injections such that a repeating order, standard, sample, standard, is achieved. Peak areas and retention times were recorded with a HP 3354A data system. Percent compositions were calculated by means of peak area comparisons of samples to bracketing standards and are an average of triplicate determinations.

Results and Discussion

In conjunction with the LiChrosorb RP-18 reverse phase column, 3 solvent systems were studied during the present investigation. The solvent systems used, together with the separation characteristics achieved, are outlined in Table 1. Initial work on the chlorophenols using solvent system 2 (Table 1) resulted in insufficient resolution of the 2,4,6- and 2,4,5-TCP isomers. However, by adjusting the concentration of NaH_2PO_4 buffer from 0.3 to 0.03M, the pH from 4.4 to 5.45, and optimizing the flow rate to 2.7 mL/min (solvent system 1), a 3-min separation was obtained between the 2,4,6-/2,4,5-TCP isomers (Figure 1). Under these conditions, where the mobile phase was at pH 5.45, 2,4-D acid was not retained and eluted at the solvent front. Lack of retention permitted injections of large quantities of technical 2,4-D acid sample without adverse effects to the reverse phase column and without interference with the early eluting monochlorophenols. As expected, lowering the pH to the 2–3 region resulted in some retention and tailing of 2,4-D acid due to ionic suppression, and this consequently interfered with the lower level

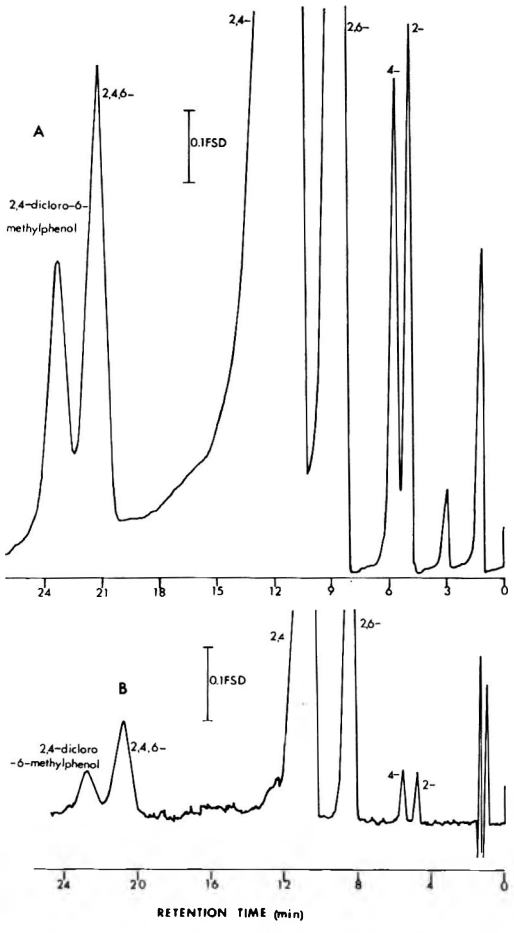

Figure 1. Separation profile of chlorophenols in a technical 2,4-dichlorophenol sample. A, electrochemical detection; B, UV detection at 280 nm. Mobile phase: 35% CH_3CN-0.03M buffer, pH 5.45; at 2.7 mL/min.

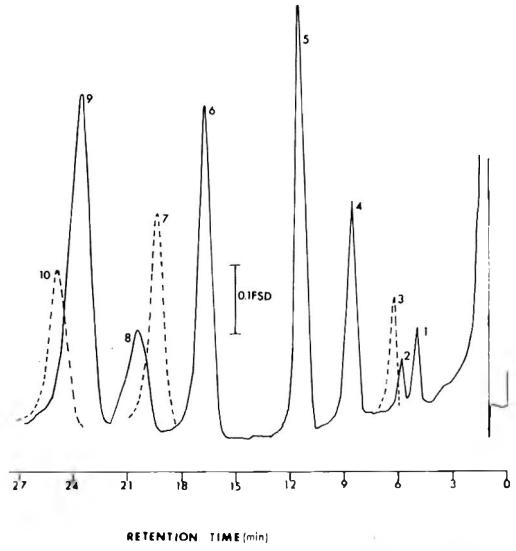

Figure 2. Composite chromatogram showing separation of 10 chlorophenol standards, using electrochemical detection. Mobile phase: See Figure 1. Peak identification: (1) 2-chlorophenol, (2) 4-chlorophenol, (3) 3-chlorophenol, (4) 2,6-dichlorophenol, (5) 2,4-dichlorophenol, (6) 2,3,6-trichlorophenol, (7) 2,3,4-trichlorophenol, (8) 2,4,6-trichlorophenol, (9) 2,4,5-trichlorophenol, (10) 2,3,5-trichlorophenol.

quantities of the mono- and dichlorophenol impurities that were present. Use of a Partisil ODS column and an acetonitrile-0.3M buffer eluant at pH 2.95 resulted in 2,4-D appearing in the "window" between the 2,4- and 2,6-dichlorophenol impurities (3). For the isomer-specific assay of 2,4-D acids a third solvent system was employed (solvent system 3, Table 1). Although 2,4-D acid eluted in 9 min, the retention time of 2,4-DCP was exceptionally long at 27.5 min. Therefore, for quantitative analysis of the chlorophenols in technical 2,4-DCP and 2,4-D acids, solvent system 1 was used; solvent system 3 was used only for the isomer-specific assay of 2,4-D acid.

For quantitative analysis of 2,4-D, the UV wavelength of choice in previous reports (3, 4, 7, 8) has been 280 nm. However, sensitivity is greater at 208 nm (Figure 2), but so also are the number of interference peaks encountered greater (Figure 3). Sensitivity at 208 nm for 2,4,6-TCP is similar to that observed with the electrochemical detector operating at a potential of 0.85 V (Figure 4). Quantitative determinations of chlorophenols at 280 nm, which is commonly used with fixed wavelength HPLC detectors, is about 10 times less sensitive than the electrochemical detector. This can be seen in Table 2 which gives the minimum detectable amounts for 7 chlorophenols. In the present study, quantitative analysis was performed on the electrochemical detector with confirmation at 208 nm on the UV detector, both detectors being connected in series.

Table 3 outlines the results obtained from the analysis of 10 technical samples of 2,4-dichlorophenol (2,4-DCP). As expected, the major impurities were the mono- and tri-chlorinated phenols arising from under- and over-chlorination steps, together with the 2,6-dichlorophenol isomer. The average 2,4-DCP content of these technical materials was 92.24%. The major side-products, *o*- and *p*-chlorophenol, 2,6-dichlorophenol together with 2,4,6-trichlorophenol amounted to 7.27%, the remaining 0.49%

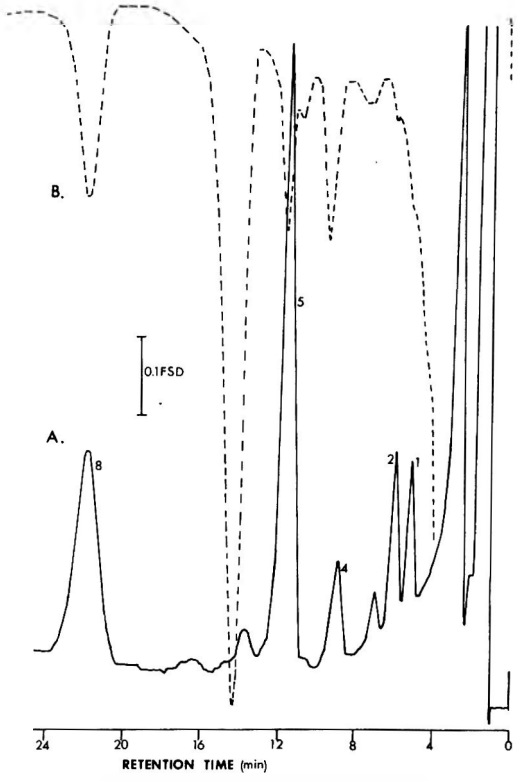

Figure 3. 2,4-D acid sample chromatograms: A, UV detection at 208 nm; B, electrochemical detection. Peak identification: See Figure 2.

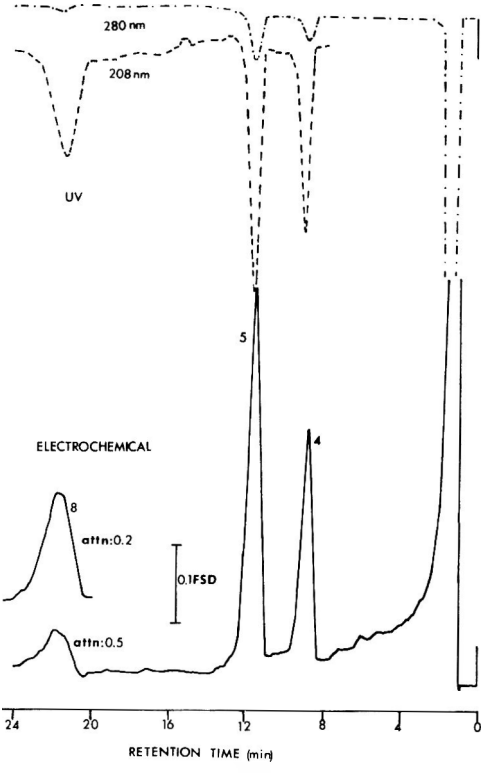

Figure 4. Chromatograms of standard containing 2,4-di-, 2,6-di-, and 2,4,6-trichlorophenols, with UV detection at 208 nm and with electrochemical detection. Mobile phase: See Figure 1. Peak identification: See Figure 2.

content being ascribed to HCl or water residue from the phenol chlorination reaction. Initial analyses of samples V and X (Figure 1) revealed that the peak appearing after 2,4,6-TCP had the identical retention time of 2,4,5-TCP. However, since this tentative finding appeared unlikely, the compound was collected, after separation using a silica gel column, methylated using diazomethane and compared with a methylated 2,4,5-TCP standard by FID-GC and found to be different. Mass spectral analysis (Figure 5) showed the compound had, in fact, not been methylated, and it was identified as 2,4-dichloro-6-methyl phenol, both by empirical formula determination and retention time comparisons (GC and HPLC) using an authentic standard. This impurity has also been found in MCPA (9). The level of 2,4-dichloro-6-methyl phenol in samples V and X was 0.34%, and its presence in 2,4-DCP can be viewed as a possible cross-contamination problem.

With prolonged use, the LiChrosorb RP-18 column became inefficient. Peak splitting occurred with 2,6-DCP, and irregular shaped peaks were observed for 2,4,6-TCP, and 2,4-dichloro-6-methyl phenol. However, increased column life and sharper peaks were obtained by varying the ionic strength of the mobile phase from 0.03 to 0.09M (Table 1).

In only 4 samples (Nos. II, III, IV, VII) was no 2,4,6-TCP found and these were also the samples exhibiting the highest percent 2,4-DCP. The

Table 2. Minimum detectable amounts [a] of various chlorophenols

Compound	UV, 280 nm	Electrochem., 0.85 V
o-Chlorophenol	10	1
p-Chlorophenol	15	1
m-Chlorophenol	10	1
2,6-DCP	20	2
2,4-DCP	20	2
2,4,6-TCP	40	8
2,4,5-TCP	20	2
2,4-Dichloro-6-methyl phenol	20	4

[a] Expressed in ng giving S/N = 2.

Table 3. Determination of free phenols (%) in dichlorophenol

Sample No.	*o*-Chloro-phenol	*p*-Chloro-phenol	*m*-Chloro-phenol	2,4-Di-chloro-phenol	2,6-Di-chloro-phenol	2,4,6-Tri-chloro-phenol	2,4-Dichloro-6-methyl-phenol	Phenol, total
I	1.08	0.72	ND[a]	87.41	3.65	1.75	ND	94.61
II	0.35	0.08	ND	98.20	3.60	ND	ND	102.20
III	0.10	0.57	ND	98.30	1.40	ND	ND	100.37
IV	0.23	0.05	ND	96.39	3.32	ND	ND	99.99
V	0.19	0.20	ND	94.00	4.38	1.46	0.34	101.05
VI	1.46	0.36	ND	86.22	5.64	3.23	ND	96.91
VII	0.17	0.20	ND	97.64	1.89	ND	ND	99.90
VIII	3.35	0.82	ND	86.19	7.12	2.91	ND	100.40
IX	3.52	1.19	ND	83.90	9.18	2.53	ND	100.32
X	0.43	0.37	ND	94.11	4.60	0.53	0.34	100.38
Range	0.10–3.52	0.05–1.19	ND	83.90–98.30	1.40–9.18	0.53–3.23	—	94.61–102.20
$\bar{X}$	1.09	0.46	—	92.24	4.48	1.24	0.07	99.61

[a] ND = not detected.

percent free phenols found in 13 technical 2,4-D acids are given in Table 4. These were the same as found in technical 2,4-DCP, with 2,4-DCP itself the major chlorophenol impurity. Except for samples 8 and 9 the average chlorophenol content was 0.08%. In samples 8 and 9 exceptionally high 2,4- and 2,6-DCP levels were found and may reflect thvariation that can be expected in the 2,4-D acid processes employed by the various manufacturers. A comparison between the electrochemical and 208 nm UV detector responses (for a typical 2,4-D acid) (sample 11) is

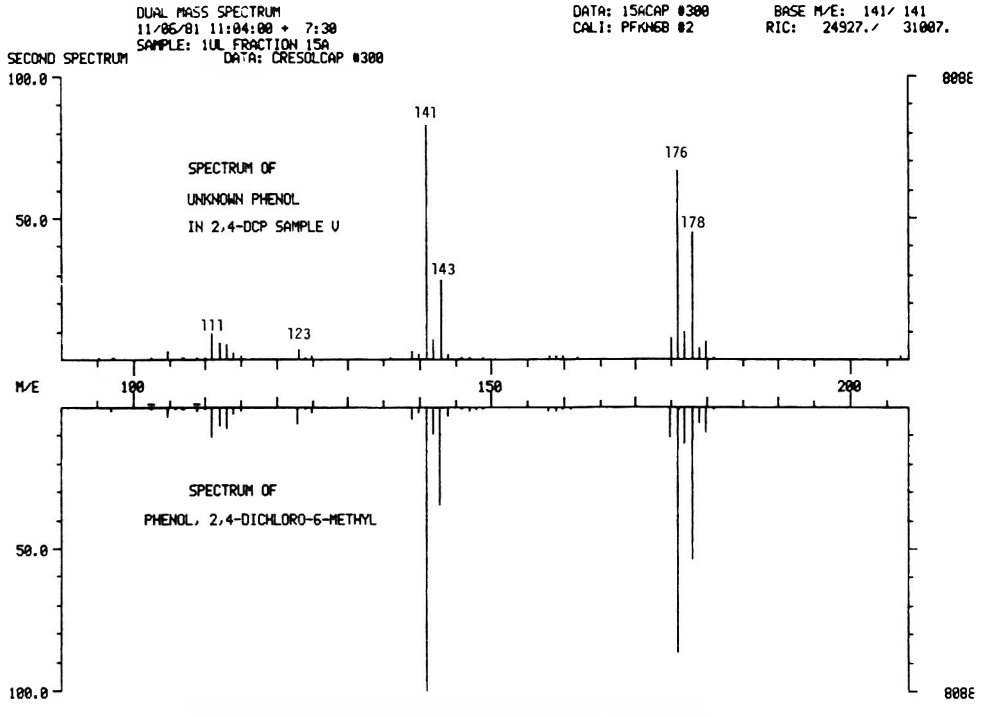

Figure 5. Mass spectral comparison of 2,4-dichloro-6-methyl phenol with that isolated from 2,4-DCP sample: GC/MS on Finnigan 4025 instrument with INCOS data system and 3% SE-30 Ultraphase column at 185°C.

Table 4. Determination of free phenols (%) in technical 2,4-D acid [a] from different sources

Sample	*o*-Chloro-phenol	*p*-Chloro-phenol	2,6-Dichloro-phenol	2,4-Dichloro-phenol	2,4,6-Tri-chloro-phenol	Acids	Total
1	0.0021	0.0008	0.02	0.063	0.006	95.0	95.0919
2	0.0007	<0.0005	0.01	0.023	0.03	96.81	96.874
3	0.0007	<0.0005	<0.001	0.004	<0.001	99.29	99.297
4	0.0009	<0.0005	0.002	0.017	0.006	97.6	97.626
5	0.0014	0.0018	0.002	0.036	<0.001	96.55	96.592
6	<0.0004	<0.0005	0.02	0.030	0.02	89.77	89.841
7	0.0019	0.0006	0.02	0.050	0.01	92.01	92.092
8	<0.0004	<0.0004	0.01	1.45	<0.005	91.17	92.636
9	0.004	0.005	0.048	0.582	0.14	94.18	94.960
10	0.0012	0.0004	0.016	0.056	0.0065	95.08	95.16
11	0.0015	0.0021	0.002	0.0180	0.031	97.59	97.646
12	0.0011	0.0011	0.024	0.110	0.022	93.90	94.059
13	0.00143	0.00098	0.026	0.061	0.0079	95.39	94.488
Range	0.0004–0.004	0.004–0.005	0.001–0.048	0.004–1.45	0.001–0.14	89.77–99.29	89.841–99.297
$\bar{X}$	0.001	0.001	0.01	0.19	0.02	94.949	95.105

[a] *m*-Chlorophenol and 2,4-dichloro-6-methyl phenol were not found in any sample.

illustrated in Figure 3. As can be seen, major extraneous peaks are evident at 208 nm, which are non-phenolic in nature because a corresponding response was not observed in the EC chromatogram.

Total acid content is also given in Table 4 and this varied from 89.77 to 99.29%. These figures do not change drastically when the free chlorophenols are taken into consideration. Therefore, the variation of 5% from the average 2,4-D acid content of 95% can only, as before, be attributed to the presence of NaCl and water. In samples 2, 4, and 11 (Table 4) the water content varied from 1.9 to 3.0% while NaCl content ranged from 40 ppm to 0.3%.

The HPLC procedure with electrochemical detection described in this paper provides a quick and facile method for the determination of 5 chlorophenol impurities still remaining in technical chlorophenol and phenoxy acids. Also, it provides an alternative to the existing isomer-specific HPLC methods for chlorophenoxy acid formulations (3, 4, 8, 9).

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TECHNICAL COMMUNICATIONS

Portable Micro Method for Quantitative Determination of Vitamin C in Fruit and Vegetable Juices

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A portable rapid and accurate micro method is described for the quantitative determination of vitamin C in fruit and vegetable juices. Chromatographic paper is impregnated with DCP by immersion in 95% ethanol solution and sprayed with a protective starch solution. Papers are usable indefinitely when protected from moisture, light, air, and high temperatures. Determinations can be completed in 60 s with an accuracy of ±2.0 mg/100 mL fruit or vegetable juice. Cloudy or lightly colored samples do not interfere with the determination. The method was compared with the official AOAC 2,6-dichloroindophenol (DCP) titration method and gave a linear correlation coefficient of 0.99749.

The 2,6-dichloroindophenol (DCP) titration method (1) is the official AOAC method for assay of vitamin C in fruit and vegetable juices. However, several aspects of the method could be improved. At the end point of the titration, excess unreduced dye appears as a pale rose pink which slowly fades and must be timed to persist for ≥5 s. Most fruit and vegetable juices retain some color or cloudiness after filtration which tends to mask the pale pink of the end point; considerable experience is required of the analyst to obtain consistent results. The amount of sample required (to contain 2 mg ascorbic acid) for the test necessitates the time-consuming filtration of large volumes of many fruit and vegetable juices of low vitamin C content.

The objectives of the present study were to reduce the difficulties with the titration method end point, to shorten the total time for analysis, to use micro amounts of sample, and to increase the portability of the test.

METHOD

Reagents and Apparatus

(a) *2,6-Dichloroindophenol.*—(Fisher Certified, ACS No. S-296).

(b) *Chromatographic paper.*—Whatman No. 1 (46 × 57 cm).

(c) *Spray starch.*—Niagara spray starch, No. 4 J31-P4B (Best Foods Div., CPC International, Inc., Indianapolis, IN 46206), or equivalent.

(d) *Glass atomizer.*—125 mL (Fisher 05-119-5C).

(e) *Buffer.*—Add 70.0 g citric acid monohydrate to 420 mL 1N NaOH and dilute to 1 L. Adjust as necessary with acid or base to pH 3.5. Store in refrigerator.

(f) *Standard ascorbic acid solutions.*—Weigh 100 mg USP Reference Standard Ascorbic Acid that has been stored in desiccator. Transfer to 100 mL volumetric flask and dilute to volume with buffer. Freshly prepare serial dilutions by accurately pipetting (±0.01 mL) appropriate amounts into 10 mL volumetric flasks and diluting to volume with buffer. Use concentrations of 40, 50, 60, 70, 80, 100 mg/100 mL for 0.1% test papers; 24, 28, 32, 36, 40, 44 mg/100 mL for 0.05% test papers; and 4, 8, 12, 16, 20, 24 mg/100 mL for 0.025% test papers.

(g) *Disposable micro pipets.*—Microcaps 10 μL (Drummond Scientific Co.).

(h) *TLC lacquer.*—Camag lacquer No. 2732M65 (Arthur H. Thomas Co., Philadelphia, PA).

Preparation of Test Papers

Prepare three 1 L solutions of DCP in 95% ethanol in concentrations of 0.1, 0.05, and 0.025% w/v. Store in refrigerator until used for impregnating chromatographic papers. Solutions can be stored for extended periods under refrigeration but should be discarded when papers are visually lighter in color than the original paper. To prepare a quantity of test papers, titrate solutions against ascorbic acid standard and replenish DCP to original concentrations. Cut papers (b) into strips 11 × 57 cm and immerse in DCP solutions in glass trays (ca 5 × 20 × 35 cm) for 3 min with continuous agitation. Remove strips from trays and rotate during draining to ensure even coverage of dye. Hang strips on glass rods in exhaust hood until dry, and immediately spray both sides of papers to point of saturation with spray starch, using a glass atom-

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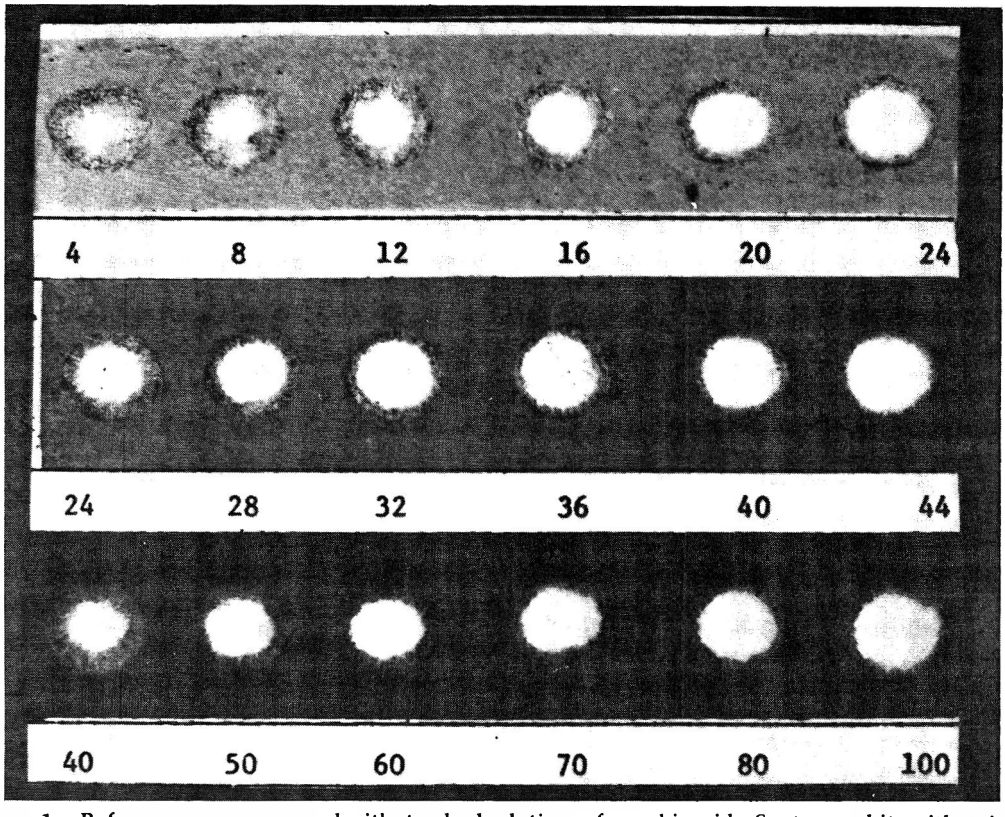

Figure 1. Reference papers prepared with standard solutions of ascorbic acid. Spots are white with a pink ring on blue paper.

izer. As soon as papers are dry, cut into 2.5 × 11 cm test papers and store in clean, dry, brown jars away from light. When a test paper is removed from a jar for any reason, do not return it to the jar.

Preparation of Reference Papers

Prepare fresh standard L-ascorbic acid solutions. Spot strips using 10 μL disposable pipets. Reference paper spots do not fade for up to 2½ h, but if reference papers are to be used for longer periods (up to 8 h), spray with TLC lacquer. Spots are white at center with a pink ring on blue background, Figure 1.

Spot Test Procedure

Prepare fruit or vegetable juice samples as described in *Official Methods of Analysis* (2) with the following modifications: Only a few drops of sample are required for spot test instead of the volumes required for titration method, and citrate buffer replaces metaphosphoric-acetic acid buffer. When filtering processed juices or collecting fresh juices in the field as described earlier (4), discard first 10 drops of filtrate before collecting a few drops for spot test. If approximate range of vitamin C content of juice sample is known, apply 10 μL full strength juice or juice diluted with buffer to either 0.05% test paper (range 24–40 mg/100 mL), or to 0.025% test paper (range 4–24 mg/100 mL). If vitamin C content of juice sample is unknown, use 0.1% test paper first as follows: Apply 10 μL full strength juice to 0.1% test paper. If spot is solid pink, indicating that vitamin C content is <40 mg/100 mL, use 0.05% test paper next. If spot on 0.05% test paper is also solid pink, sample vitamin C content is <24 mg/100 mL and within range of 0.025% test papers. If spot on 0.1% test paper is all white, indicating that C content is >100 mg/100 mL, dilute juice with buffer until spot produced on 0.1% test paper is white with pink ring on blue paper (Figure 1). Compare area of white spot and width of pink ring with corresponding parts of spots on reference papers to assign vitamin C concentration equal to one of reference spots or halfway between two, with

Table 1. Comparison [a] of spot test with official AOAC method for fruit and vegetable juices [b]

Sample	Official, mg/100 mL			Spot, mg/100 mL		
1	16.5	15.2	15.8	16	16	16
2	45.7	46.0	45.1	44	44	44
3	66.0	64.8	66.0	64	64	64
4	15.5	14.6	14.8	16	16	16
5	55.2	55.2	56.2	56	60	56
6	35.4	35.1	36.3	36	32	36
7	55.1	55.9	54.3	56	56	56
8	40.8	40.8	41.3	40	40	44
9	61.0	61.0	60.4	60	60	56
10	32.3	31.8	32.3	32	32	32
11	46.2	45.7	44.9	44	40	44
12	34.1	34.1	34.1	32	36	36
13	41.0	40.7	41.0	40	40	40
14	39.8	39.8	39.6	40	40	40
15	22.5	19.8	18.7	24	20	20
16	NA	NA	NA	48	50	50
17	24.6	24.1	25.2	28	28	28
18	35.0	37.1	35.0	36	36	36
19	7.6	7.8	8.1	8	8	8
20	19.9	19.1	20.2	20	20	20
21	15.9	16.5	16.2	16	16	16
22	7.9	7.5	6.0	8	8	6
23	20.8	20.2	19.6	20	20	20
24	5.0	4.8	5.0	4	6	6
25	20.2	19.6	19.6	20	22	20

[a] Linear correlation coefficient for test comparison was 0.99749, excluding Sample 16 for which no determinations could be made by AOAC method because of pink color of sample.

[b] Food samples were purchased from several local grocery stores on several different dates: (1) V-8 Vegetable Juice; (2) Hi-C Apple Drink; (3) Orange-Plus Frozen Breakfast Beverage; (4) Campbell's Tomato Juice; (5) Frosty Acres Frozen Orange Juice; (6) Frosty Acres Frozen Grapefruit Juice; (7) Minute Maid Frozen Orange Juice; (8) Hi-C Apple Drink; (9) DelMonte Pineapple-Orange Drink; (10) Hy-Top Unsweetened Grapefruit Juice; (11) Minute Maid Frozen Orange Juice; (12) Tang Instant Breakfast Drink; (13) Lucky Leaf Apple Juice; (14) Hi-C Orange Drink; (15) fresh tomatoes; (16) Ocean Spray Cranapple Juice; (17) Thrifty Maid Frozen Strawberries; (18) fresh lemons; (19) fresh celery; (20) DelMonte Mandarin Oranges; (21) Green Giant Sweet Peas; (22) fresh Irish potatoes; (23) DelMonte Whole Peeled Tomatoes; (24) Frosty Acres Frozen Green Beans; (25) Frosty Acres Frozen Mustard Greens.

accuracy of ±5.0 mg/100 mL for 0.1% test papers. Repeat determination with either 0.05% test paper or 0.025% test paper, for accuracy of ±2.0 mg/100 mL, by diluting samples with buffer to bring vitamin C content within ranges of one of these test papers. For example, if 0.1% test paper result indicates vitamin C content between 50 and 80 mg/100 mL, a 1:1 dilution will bring sample within range of 0.05% test paper.

Results and Discussion

Impregnation of chromatographic papers with water or buffer solutions of DCP was unsuccessful because the dye changed from blue to pink by the time the papers were dry. Ethanol solutions dried rapidly and possibly the crystalline structure of the dye was altered so that the blue color was preserved. However, without starch coating, the color of papers even when sealed in glass jars away from light degraded within 7 h (4). Papers sprayed with commercial spray starch retained essentially the same color values for 60 days at room temperature, and color photographs of reference papers were useful as references for this period. Starch-coated papers can be kept in brown bottles and used for years with freshly prepared reference strips even though there is a gradual change of color.

Spotting of reference strips or test papers is simple as long as care is taken to ensure that the sample is quantitatively transferred from the microliter pipet to the test paper. The tip of the pipet is touched to the paper and capillary action draws the sample onto the paper with very little pressure of the pipet bulb needed. Uniform spotting can be accomplished with a minimum of practice. Reduction of the dye proceeds to completion rapidly and spots can be read as soon as they are dry. If papers are prepared in advance with identifying marks, at least 15 samples can be spotted in the time interval necessary for one titration, and the first spots will be dry by the time the last sample is spotted. Triplicate spots of most samples are usually evaluated identically,

unless excess pigment or pectin content introduces a deviation of ±2.0 mg/100 mL. If the sample has been diluted 1:1 with buffer, the maximum deviation will be 4.0 mg/100 mL.

Samples for comparison of the spot test with the official AOAC titrimetric method were purchased from local grocery stores and both tests were conducted on the same day (Table 1). The canned, bottled, or frozen juices were usually spotted full strength on the 0.1% test papers and then diluted 1:1 with buffer solution to bring them into the range of either the 0.05 or the 0.025% paper. When papers were spotted with samples diluted 1:1 and triplicate readings varied by 2 mg/100 mL, the multiplication factor produced a variation of 4 mg/100 mL. The average standard deviation for the spot test results was 2.3 compared with 1.9 for the titration method; however, the linear correlation between the 2 methods was 0.99747. Some samples with residual pink color such as No. 16 (cranberry-apple) could not be determined by the titration method but were analyzed by the spot test. Concord grape juice could not be analyzed by either test.

The spot test is useful for any sample for which the AOAC titration method is valid. The spot test is so much faster that 10 or more test (spots) can easily be applied whenever indecision in comparisons with the reference spots occurs, and thus variation can be reduced. Interfering ions in samples should be determined using methylene blue (1) to see if the method is valid for the sample, or the samples can be treated (3) to remove the interfering ions. The micro amounts of sample required greatly reduce the time and labor involved in preparation of samples, making the method especially useful in determination of vitamin C in individual fruits and vegetables. Freshly prepared reference strips are recommended for highest accuracy, but actual-size colored photographs of freshly prepared reference strips increase the portability of the method, and are accurate for extended periods (4). Cloudy or lightly colored samples do not interfere with the determination and only a minimum of skill is required of the analyst.

REFERENCES

(1) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs. **43.056-43.060**

(2) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, sec. **22.008**

(3) Pelletier, O., & Morrison, A. B. (1966) *J. Assoc. Off. Anal. Chem.* **49**, 800-803

(4) Rymal, K. S., & Rice, C. A. (1976) *Hortic. Sci.* **11**, 23-25

Procedure for Cooking Seafood Products

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As a result of experience gained using procedure 18.003, a revision is proposed. The revised procedure has been adopted.

In 1975, AOAC accepted procedure **18.003** for cooking seafood products (1). Its objective is to standardize techniques for heating to prepare seafood products for grading the quality of their flavor and odor. It is based on uniform directions published in U.S. Standards for Grades of certain seafood products. It has also been used successfully to inspect seafood products for which U.S. Standards for Grades have not yet been developed.

Under the Joint FAO/WHO Food Standards Program, the Codex Alimentarius Commission and, in particular, its Committee for Fish and Fishery Products have been incorporating procedure **18.003** in standards which are used in international trading. The following documents include procedure **18.003**:[1]

Recommended International Standard for

[1] Recommended International Standards are published by the Secretariat of the Joint FAO/WHO Food Standards Program, FAO, Via delle Terme di Caracalla, 00100 Rome, Italy, and distributed by FAO sales agents in member countries. The U.S. sales agent is UNIPUB, Inc., 345 Park Ave S, New York, NY 10010.

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The recommendation of the Associate Referee was approved by the General Referee and Committee C and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, 346; 417.

Quick Frozen Fillets of Flat Fish, CAC/RS 91-1976

Recommended International Standard for Quick Frozen Shrimps or Prawns, CAC/RS 92-1976

Recommended International Standard for Quick Frozen Fillets of Hake, CAC/RS 93-1978

Recommended International Standard for Quick Frozen Lobsters, CAC/RS 95-1978

Proposed Draft Standard for Quick Frozen Blocks of Fish Fillets, Minced Fish Flesh, and Mixtures of Fillets and Minced Fish Flesh

Proposed Draft Standard for Quick Frozen Fish Sticks (Fish Fingers) and Fish Portions—Breaded or in Batter

The purpose of this communication is to describe experience obtained since 1975 and to propose that procedure **18.003** be revised. To measure internal temperature, the most practical instrument has proven to be a thermocouple mounted inside a rigid tube with an adjustable probe and a temperature read-out. Such instruments are commercially available at a reasonable cost. We have also received comments that a statement should be added to the deep fat frying procedure saying that cooking oil should be changed frequently so that heat-induced changes in the composition of cooking oil, which has been used for a long time, will not interfere with sensory evaluation of a cooked product.

The usefulness of procedure **18.003** has been limited by the fact that it describes only 4 techniques of heating: baking, boil-in-bag, deep fat frying, and steaming. In the following paragraphs, some examples of these limitations are given.

On some occasions, cooking directions are given on the packages of a sample being inspected. Such directions are more common on packages intended for direct sale at a retail level. They are used infrequently on packages intended for the food service trade or for sale at an institutional level. When cooking directions are given, they might be used during an official inspection. To standardize heating techniques between samples of different origins, 2 requirements should be met. The directions should provide thermal conditions equivalent to procedure **18.003** (heating product to an internal temperature of at least 160°F (70°C) and they should not describe substances (other than cooking oil for frying) that would alter the natural flavor and odor of the cooked product. If cooking directions are given in a language that an inspector does not understand or if no cooking directions are given, an inspector would use procedure **18.003**.

The U.S. Army Natick Research and Development Laboratories has completed a recent study under a contract with the National Marine Fisheries Service entitled "Consumer and Instrumental Edibility Measures for Grouping of Fish Species" (2). One of the objectives of this study is to develop a standardized methodology which can be used by other laboratories in their assessments of other species of fish. At the beginning of this study, the cooking procedures in procedure **18.003** were tried. The boil-in-bag procedure was the best choice but it did not provide a sufficiently reproducible heat treatment for the purposes of this study. The adopted procedure was to package a piece of fresh fish fillet in a sealed 2-compartment boilable film-type pouch and to cook it by immersion in 160°F (70°C) water. The time required for cooking was determined by placing thermocouples at the center of samples of different thicknesses and then performing heat penetration studies. Cooking was completed when the coldest part of a sample reached 160°F (70°C).

In the development of a new U.S. Standards for Grades of Fresh or Frozen Fish Steaks (50 CFR 262, Subpart A), broiling has been suggested as a cooking procedure. Broiling and baking are commonly used for this product but only baking is presently described in procedure **18.003**.

In the development of a Proposed Draft Standard for Quick Frozen Fish Sticks (Fish Fingers) and Fish Portions—Breaded or in Batter, the Codex Committee for Fish and Fishery Products is considering baking, frying in a shallow pan, deep fat frying, and grilling for sensory assessment of these products. Shallow pan frying and grilling are used for inspection purposes in some other countries but they are not described in procedure **18.003**.

Microwave ovens have been suggested for use by Inspection Agencies because they offer rapid heating, especially for precooked seafood products. They are also becoming more readily available in places where official inspection of seafood products is performed, but microwave heating is not described in procedure **18.003**.

Examples of other cooking procedures could be given but it is obvious that including all of them would make procedure **18.003** quite lengthy. Instead, we propose to omit all detailed techniques of heating a product and to focus on the requirement that a product be heated to an internal temperature of at least 160°F (70°C).

The following revised procedure is recommended for adoption.

18.D01 ***Cooking of Seafood Products Procedure***

Cooking procedure is based on heating product to internal temp. ≥160°F (70°C). Cooking times vary according to size of product and equipment used. To det. cooking time, cook extra sample same way using temp. measuring device with probe of known length to det. internal temp. Cooking equipment, including cooking oil for deep fat frying, shall be free from substances which interfere with sensory evaluation of cooked product.

Methods of heating product include, but are not limited to, baking, bake-in-foil, broiling, boil-in-bag, shallow pan frying, deep fat frying, oven frying, grilling, poaching, steaming, and microwave heating.

REFERENCES

(1) King, F. J. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 225–226

(2) Kapsalis, J. G. (1981) "Consumer and Instrumental Edibility Measures for Grouping of Fish Species," Final Report of Contract 01-8-M01-6320, U.S. Army Natick Laboratories, Natick, MA

Report of the Twenty-Sixth Annual Meeting of the Collaborative International Pesticides Analytical Council (CIPAC)

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The 26th Annual CIPAC Meeting and Symposium was held at the FAO (Food and Agriculture Organization) Headquarters in Rome, Italy, in May 1982 and was hosted by the FAO's Plant Protection Service. Arrangements were made by A. V. Adam.

The annual informal meeting of FAO and GIFAP (International Group of National Association of Agrochemicals) preceded the Symposium. An explanation of the FAO-GIFAP meeting was given in the 1981 report (Bontoyan, W. R. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 400–403). Tentative agreements on a number of specifications for technical and formulated pesticides were approved. These specifications are important to the U.S. Department of Agriculture and EPA as well as U.S. pesticide manufacturers. Information on these specifications can be obtained from Dr. A. V. Adam, the Food and Agriculture Organization of the United Nations, Via della Terme di Caracalla 00100, Rome, Italy.

H. H. Povlsen of Kemikaliekontrollen, Denmark, and M. Laurent, chairman of the GIFAP Technical Committee, co-chaired the Symposium. Among the papers presented were seven on formulation analysis. These consisted of three using high pressure liquid chromatography (HPLC), three by various assay procedures, and one on the aspects of HPLC in quality control of technical and formulated pesticides. Also presented were papers on physical testing (including testing for fire and explosion hazards of pesticides) and a presentation on Integrated Pest Management with Controlled Release Pheromone Formulations.

The organizational makeup and membership of CIPAC is essentially the same as given in the 1981 report. However, the Council elected additional Correspondents: A. V. Adam of FAO; S. Bailey of Boots Chemical, England; J. W. Miles of the U.S. Department of Health and Human Services.

The CIPAC Management Committee decided to hold the 27th CIPAC Annual Meeting and Symposium and the FAO-GIFAP Meeting at Griffin University, Brisbane, Australia, from July 5 to 14, 1983. CIPAC also agreed to hold its 28th Annual Meeting (1984) in the United States in conjunction with AOAC's Centennial Meeting.

The summaries of the decisions taken at the 26th meeting of the Technical Committee of CIPAC in Rome, Italy, May 24 and 25, 1982, are listed below. Seven AOAC methods for pesticide formulation analysis were adopted by CIPAC.

Code Number	*Chemical*	*Status of the Method*
1	2, 4-D	The GLC method, MT 129-CIPAC/3030/(M) for the determination of 2,4-D technical was adopted as provisional CIPAC method.
10.a	parathion-methyl	The GLC method for parathion-methyl encapsulated formulations, **6.409-6.414,** was adopted as full AOAC-CIPAC method.
32. + 33	pyrethrins + piperonyl butoxide	The GLC method for pyrethrins + piperonyl butoxide, **6.C22-6.C25,** was adopted as provisional AOAC-CIPAC method.
40	captan	The GLC method for the determination of captan in captan technical and formulations, **6.215-6.219,** was adopted as full AOAC-CIPAC method. The HPLC method for the determination of captan in captan technical and formulations, **6.A09-6.A14,** was adopted as full AOAC-CIPAC method.
45	mevinphos	The HPLC method for the determination of mevinphos technical and formulations, CIPAC-P81, pp. 283–288, was adopted as full CIPAC method.
59	dimethoate	The TLC method and the GLC method, CIPAC/3026/M, for the determination of dimethoate in dusts, wettable powders, and granules were adopted as full CIPAC methods.
77	phenmedipham	The TLC-densitometric method for the determination of phenmedipham in emulsifiable concentrates, CIPAC-P 80, pp. 215–219, was adopted as full CIPAC method. The HPLC method and the titrimetric method, presented at the CIPAC Symposium 1982, for the determination of phenmedipham in emulsifiable concentrates were adopted as provisional CIPAC methods.
78	quintozene	The GLC method for the determination of quintozene in quintozene technical and formulations, **6.C08-6.C11,** was adopted as provisional AOAC-CIPAC method.
83	2,4-DB	The GLC method for the determination of 2,4-DB technical, MT 129-CIPAC/3030/(M), was adopted as provisional CIPAC method.
84	dichlorprop	The GLC method for the determination of dichlorprop technical, MT 129-CIPAC/3030/(M), was adopted as provisional CIPAC method.
87	bromoxynil octanoate	The GLC method, CIPAC/3006/M, for the determination of bromoxynil octanoate technical was adopted as full CIPAC method.
118	fenoprop	The GLC method, MT 129-CIPAC/3030/(M), for the determination of fenoprop technical was adopted as provisional CIPAC method.

Code Number	*Chemical*	*Status of the Method*
172	chinomethionat	The HPLC method, CIPAC/3040/(M), for the determination of chinomethionat technical and formulations was adopted as provisional CIPAC method.
221.b	chlorpyriphos	The HPLC method, **6.B15-6.B19,** for the determination of chlorpyriphos technical and formulations was adopted as full AOAC-CIPAC method.
263	carbendazim	The HPLC and UV spectrophotometric methods, CIPAC-P 81, pp. 144–151, for the determination of carbendazim technical and formulations were accepted as full CIPAC methods.
353	triazophos	The HPLC method, CIPAC-P 81, pp. 324–331, for triazophos technical concentrates and emulsifiable concentrates was adopted as full CIPAC method.
358	diclofop-methyl	The GLC method for the determination of diclofop-methyl technical and emulsifiable concentrates, CIPAC/3044/(M), was adopted as provisional CIPAC method.
360	triforine	The HPLC method for the determination of triforine in emulsifiable concentrates, CIPAC-P 81, pp. 341–345, was adopted as full CIPAC method.
MT 47.2		The method for the determination of foaming of suspension concentrates, CIPAC/3028/R, App. A, was adopted as full CIPAC method.
MT 157		The method for the determination of water solubility of substances with solubilities higher than 10^{-2} g/L, CIPAC/3053/M, was adopted as full CIPAC method.

FOR YOUR INFORMATION

Report of the Committee on Statistics

The Committee's report was accepted at the Business Meeting on October 28, 1982, during the 96th Annual International Meeting of the AOAC. Committee members are: James S. Winbush (Food and Drug Administration, Washington, DC 20204), *Chairman;* Charles Annello; Paul R. Caudill; D. Earle Coffin; Edwin Glocker; Patrick C. Kelly; Robert C. Rund; Marie Siewierski.

The Statistics Committee discussed problems in recent collaborative studies and suggestions for improved analysis and interpretation. William Horwitz, Chairman of the Committee on Collaborative Studies, discussed some statistical problems encountered by his committee. One problem, discussed at length, concerned the pros and cons of an overall analysis of variance and the analysis of the collaborative data on a sample-by-sample basis. Also discussed were the biases associated with the use of the Youden ranking procedure for determining whether a laboratory is an outlier. It was decided that the Youden rank procedure should be used with discretion, recognizing that the procedure is not always effective in determining a true outlier.

Mini-collaborative studies were discussed, questioning what type of sanction can be given to them and recognizing that such studies fill a need when minor modifications are made in a method.

There are problems associated with the practice of not performing the official method at the same time that the newer method is being collaboratively tested. Due to greater constraints on funds and collaborators' time, this practice has been increasing.

The Committee discussed various aspects of making available a computer program to perform part of the statistical analyses for collaborative studies. The program was developed and modularized by members of the FDA Division of Mathematics. While the program might not be used properly on occasion, its overall benefits outweigh this negative aspect because statisticians are available for consultation.

Papers or items relative to statistical issues in collaborative studies will be submitted to the *Referee* or the *Journal*.

New Additions to the AOAC Sustaining Membership List

AOAC is pleased to announce that the Laboratory of the Government Chemist, London, England, as a Sustaining Member, and Merck Sharp and Dohme Research Laboratories, Rahway, NJ, as a Private Sustaining Member, have joined the list of organizations that support independent methods validation. The support of all Sustaining Members helps further the purposes of AOAC.

New NBS Standard Reference Materials Recently Made Available

The National Bureau of Standards Office of Standard Reference Materials announced the availability of the following SRMs:

SRM 1581 Polychlorinated Biphenyls in Oil—The Certificate of Analysis for this reference material lists the certified concentrations of Aroclors 1242 and 1260 present individually at 100 μg/g in motor oil and transformer oil. Additional base oil is supplied to dilute the 4 certified concentrates of PCBs. Available for $150 per unit, consisting of 8 ampules: 2.5 mL solution in 2 ampules for each of the 4 concentrates.

SRM 1599 Anticonvulsant Drug Level Assay Standard—Certified for concentrations of valproic acid and carbamazepine in a freeze-dried human serum base. Available for $153 per unit of 4 vials.

SRM 1649 Urban Dust/Organics—Atmospheric particulate matter obtained from an urban area and certified for fluoranthene, benz[a]anthracene, benzo[a]pyrene, benzo[ghi]-perylene, and indeno[1,2,3-cd]pyrene. Information values are also given for 28 inorganics and leachable anions. Available for $175 per 10 g unit.

SRMs 1805 and 1806 Benzene in Nitrogen—Certified cylinders of nominal concentrations of benzene: SRM 1805 contains 0.25 μmole/mol (ppm) and SRM 1806 contains 10 μmole/mol (ppm). Supplied in aluminum cylinders at 12.4 MPa (1800 psi) pressure with a deliverable volume of 0.88 cu. m (31 cu. ft) at STP. The cylinders conform to Dept of Transportation Specifications. The certificate issued gives the certified concentration of

benzene in each cylinder. Available for $753 per SRM cylinder.

SRM 1911 Benzene Permeation Device (Individually Calibrated)—Certified for benzene permeation rate of 0.3 to 0.5 μg/min at constant 25°C. Each device is supplied with an individual Certificate of Calibration that contains instructions for working with the certified value of that device. Permeation rates between 20 and 30°C may be calculated from data provided. Available for $296 per device.

ISO Standards Available from ANSI

The following is a list of standards from the International Organization for Standardization (ISO), Technical Committee 34-Agricultural Food Products. The Standards are available at prices indicated from American National Standards Institute, Inc., 1430 Broadway, New York, NY 10018; 212/354-3300.

ISO 676-1982	Spices and condiments—Nomenclature	$23.00
ISO 762-1982	Fruit and vegetable products—Determination of mineral impurities content	7.80
ISO 927-1982	Spices and condiments—Determination of extraneous matter content	8.00
ISO 1026-1982	Fruit and vegetable products—Determination of dry matter content by drying under reduced pressure and of water content by azeotropic distillation	9.75
ISO 1208-1982	Spices and condiments—Determination of filth	12.00
ISO 1955-1982	Citrus fruits and derived products—Determination of essential oils content (Reference method)	11.00
ISO 1956/1-1982	Fruits and vegetables—Morphological and structural terminology—Part 1	29.00
ISO 1990/1-1982	Fruits—Nomenclature	20.00
ISO 1991/1-1982	Vegetables—Nomenclature	17.00
ISO 3093-1982	Cereals—Determination of falling number	11.70
ISO 5497-1982	Sensory analysis—Methodology—Guidelines for preparation of samples for which direct sensory analysis is not feasible	8.00
ISO 5507-1982	Oilseeds—Nomenclature	21.00
ISO 5512-1982	Sunflower seed for the manufacture of oil—Specification	8.00
ISO 5513-1982	Linseed for the manufacture of oil—Specification	8.00
ISO 5564-1982	Black pepper and white pepper, whole or ground—Determination of piperine content (Spectrophotometric method)	8.00
ISO 5565-1982	Vanilla (Vanilla fragrans (Salisbury) Ames)—Specification	15.00
ISO 5566-1982	Turmeric—Determination of colouring power (Spectrophotometric method)	8.00
ISO 6538-1982	Cassis (type China, type Indonesia and type Viet Nam), whole or ground (powdered)—Specification	9.75
ISO 6775-1982	Fenugreek, whole or ground (powdered)—Specification	11.00
ISO 6634-1982	Fruits, vegetables and derived products—Determination of arsenic content (Silver diethyldithiocarbamate	

	spectrophotometric method)	$12.00
ISO 6736-1982	Dried milk—Determination of nitrate and nitrite contents (Cadmium reduction and photometry method)	12.00
ISO 6739-1982	Whey cheese—Determination of nitrate and nitrite contents (Cadmium reduction and photometry method)	12.00
ISO 7302-1982	Cereals and cereal products—Determination of total fat content	11.00

Courses Offered

Design and Analysis of Scientific Experiments.—Massachusetts Institute of Technology will offer a one-week elementary course in Design and Analysis of Scientific Experiments, July 11–July 16, 1983. Applications will be made to the physical, chemical, biological, medical, engineering, and industrial sciences, and to experimentation in psychology and economics. The course will be taught by Professors Harold Freeman and Paul Berger. Further particulars may be obtained by writing to the Director of Summer Session, Room E19-356, Massachusetts Institute of Technology, Cambridge, MA 02139.

Microscopy.—The McCrone Research Institute of Chicago will present several courses in microscopy during their 1983 schedule. Offerings include Photomicrography (101), October 17–21; Microchemical Analysis (207), September 19–23; Scanning Electron Microscopy (402) August 15–19 and November 7–11; Identification of Small Particles (501), October 10–14; Microscopical Identification of Asbestos (508A), August 1–5 and November 7–11; Advanced Asbestos Identification (508B), August 8–10 and November 14–16. Several other courses are available. Most courses are one week in length (course 508B is 3 days in length; tuition $400). Tuition is $650 per class including the text (except course 501 for which students purchase books). For further information and to register, contact Miss Nancy B. Daerr, McCrone Research Institute, 2508 S Michigan Ave, Chicago, IL 60616; 312/842-7105.

Meetings

June 1–4, 1983: Symposium—Flavour of Distilled Beverages; Stirling University, Scotland. Contact: Dr. J. R. Piggott, Dept of Bioscience and Biotechnology, University of Strathclyde, 131 Albion St, Glasgow G1 1SD, Scotland.

June 5–8, 1983: 66th Canadian Chemical Conference, Convention Centre, Calgary, Alberta, Canada. Contact: Dr. Arvi Rauk, MCIC, Dept of Chemistry, University of Calgary, Calgary, Alberta, T2N 1N4 Canada; 403/284-6247; or The Chemical Institute of Canada, 151 Slater St, Suite 906, Ottawa, Ontario, K1P 5N3 Canada; 613/233-5623.

June 7–10, 1983: 1st International Symposium on Drug Analysis, Free University of Brussels, Brussels, Belgium. Contact: C. Van Kerchove, Société Belge des Sciences Pharmaceutiques—Belgisch Genootschap voor Pharmaceutische Wetenschappen, rue Archimedesstraat 11, B-1040 Brussels, Belgium; telephone (02) 733 98 20 est. 33.

June 12–15, 1983: 87th Annual Conference of the Association of Food and Drug Officials—Government and Industry Cooperation, Marriott Hotel, New Orleans, LA. Contact: Edward D. Greer, Co-Chairman, AFDO Conference Exhibit Committee, c/o 203 Cannon Health Bldg, 75 Davis St, Providence, RI 02908; 401/277-2833.

June 14–15, 1983: AOAC Midwest Regional Section Meeting, Ames, IA. Contact: H. Michael Stahr, Iowa State University; 515/294-1950.

June 15–16, 1983: AOAC Northwest Regional Section Meeting, Olympia, WA. Contact: H. Michael Wehr, Oregon Department of Agriculture; 503/378-3793.

June 22, 1983: ASTM Committee E-3 on Chemical Analysis of Metals "Symposium on Computers in Chemical Analysis," Kansas City, MO. Contact: Kathy Greene, ASTM Publications Division, 1916 Race St, Philadelphia, PA 19103; 215/299-5414.

June 28–29, 1983: AOAC Northeast Regional Section Meeting, Burlington, VT. Contact: Audrey Gardner, NY State Agriculture Experiment Station; 315/787-2281.

July 17–23, 1983: SAC 83—International Conference and Exhibition on Analytical Chemistry, University of Edinburgh,

Edinburgh, Scotland. Contact: P. E. Hutchinson, Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BN, UK.

July 16–23, 1983: Rapid Methods and Automation in Microbiology, Kansas State University, Manhattan, KS. Certified by the American Society for Microbiology. Contact: Daniel Y. C. Fung, Dept of Animal Sciences and Industry, Kansas State University, 1623 Anderson Ave, Manhattan, KS 66502; telephone 913/532-5575.

July 18–21, 1983: 1983 National Conference of Standards Laboratories Workshop and Symposium: Metrology—Meeting the Challenge of Change, National Bureau of Standards, Boulder, CO. Contact: Gary Davidson, Co-Chairman, 1983 NCSL Workshop and Symposium, TRW/DSSG, Bldg S, Room 2767, One Space Park, Redondo Beach, CA 90278; 213/535-1684.

July 27–30, 1983: 3rd International Conference on Instrumental Analysis of Foods and Beverages—Recent Developments in Chemistry and Technology, Corfu Hilton Hotel, Corfu, Greece. Contact: D. J. Mussinan, IFF R & D, 1515 Highway 36, Union Beach, NJ 07735; 201/264-4500.

September 6–9, 1983: 5th International Bioanalytical Forum—Chromatography of Blood and Tissue Analytes, University of Surrey, Guildford, England. Contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Surrey GU2 5UL, UK; telephone 0483-65324.

September 18–23, 1983: AOCS World Conference on Oleochemicals, Montreux Congress Center, Montreux, Switzerland. Contact: Meetings Coordinator, American Oil Chemists' Society, 508 S Sixth St, Champaign, IL 61820; 217/359-2344.

September 28–30, 1983: International Workshop: Analysis of Volatiles—New Methods and Their Applications, Chemical Dept, University of Wurzburg, Germany. Contact: P. Schreier, Chairman, Prof. of Food Chemistry, University of Wurzburg, Am Hubland, D-8700 Wurzburg, GFR.

October 3–6, 1983: 97th AOAC Annual International Meeting, Shoreham Hotel, Washington, DC. Contact: Kathleen M. Fominaya, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209; 703/522-3032.

November 14–16, 1983: 3rd International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Monte Carlo, Monaco. Contact: Shirley E. Schlessinger, Symposium Manager, 400 E Randolph, Chicago, IL 60601; 312/527-2011.

December 6–7, 1983: ASTM Committee E-11 on Statistical Methods, Sheraton Bal Harbour, Bal Harbour, FL. Contact: Bill Hulse, ASTM, 1916 Race St, Philadelphia, PA 19103; 215/299-5507.

CORRECTION

J. Assoc. Off. Anal. Chem. (1982) 65, 1429–1434, "Capillary Gas-Liquid Chromatographic Determination of Vomitoxin in Cereal Grains," by H. Cohen & M. LaPointe. On the basis of ongoing work using the published method, the correction cited below improved recoveries of vomitoxin in naturally contaminated samples:

Extraction and Cleanup

Shake 10 g sample 1 h with 200 mL chloroform–ethanol (8 + 2) + 30 mL water on wrist-action shaker. Filter mixture into 500 mL round-bottom flask. Wash filtrate with additional 50 mL solvent. Add sufficient sodium sulfate to absorb all aqueous phase. Filter through small bed of sodium sulfate into 500 round-bottom flask. Wash with additional 25 mL solvent. Evaporate organic solvent and continue as indicated on page 1430.

NEW PUBLICATIONS

Toxic Materials in the Atmosphere: Sampling and Analysis. Based on the Symposium on Toxic Materials in the Atmosphere, Boulder, Colorado, August 2-5, 1981. Edited by B. F. Himmelsbach. Published by ASTM, 1916 Race St, Philadelphia, PA 19103, 1982. 166 pp. Price: $19.95. Price for ASTM members: $15.95. STP 786. ISBN 04-786000-17.

The focus of the symposium was to review all aspects of air sampling and analysis in the areas of ambient air and workplace atmospheres. This publication contains 13 papers; covers 4 major subject categories: Industrial Hygiene Sampling and Analysis, Continuous Air Monitors and Portable Instruments, Passive Monitoring Devices, and Chemical Characterization and Analytical Methods; and describes industrial hygiene aspects and techniques, specific sampling and analytical situations, and approaches to problem-solving. The first in this series, STP 721, *Sampling and Analysis of Toxic Organics in the Atmosphere*, was published in 1981.

Manual on Water, Special Technical Publication 442A. Edited by C. E. Hamilton. Published by American Society for Testing and Materials, 1916 Race St, Philadelphia, PA 19103, 1982. 471 pp. Price: $28.50, less 20% to ASTM members. ISBN 04-442010-16.

Intended as a brief reference source, this manual offers information on water and its influence on industries in which water is used as a raw material or in conjunction with manufacturing operations. It discusses the problems arising from water use with specific details on control procedures and instructions for sampling water under various conditions. This publication contains 18 papers presented at an ASTM symposium. The new edition includes information on the characteristics of water, problem areas, and new approaches for identifying and solving problems; 4 new chapters: Production and Preservation of Ultrapure Water, Sediments in Streams and Other Water Bodies, Nuclear Water Technology, and Practices for Measurement of Radioactivity; and an expanded chapter on water quality monitoring with sections on surveillance and sample conditioning. The manual can serve as a guide to the nature of water planning, to determine the significance of the treatment being applied, and as part of the indoctrination of technologists and plant operators.

Annual Drug Data Report, Volume 4. Published by J. R. Prous, S.A., Apartado de Correos 1641, Barcelona, Spain, 1982. Price: US$90. ISBN 84-300-7293-4. Also available Vol. 2, 1980; price: US$70. Vol. 3, 1981; price: US$85. Vols 2 and 3 combined, price: US$130.

This volume is the fourth in a series established as an annual guide to drugs in different stages of research, development and marketing throughout the world. The report provides in condensed form essential drug information selected from the past year's literature, symposia, congresses, and manufacturers' communications. Over 2000 entries are presented and alphabetically arranged, providing information on generic and chemical names, code designations, Chemical Abstract Registry Number, elemental composition, molecular formula and weight, graphic formula, physicochemical properties, pharmacological actions and manufacturer; and for marketed drugs information on indications, "supplied as," dosage, proprietary names in different countries, and the year first introduced. All four volumes feature accumulative indices: Cross Index for locating drugs by proprietary, chemical, and generic names and research code numbers; Pharmacological Index for locating drugs according to their particular pharmacological group; and Manufacturer Index. Selected bibliographic references list sources of information on synthesis, pharmacology, pharmacokinetics, metabolism, toxicity, clinical trials, reviews, and monographs.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 29: Some Industrial Chemicals and Dyestuffs. International Agency for Research on Cancer, Lyon, France, 1982. Available from WHO Publications Centre USA, 49 Sheridan Ave,

Albany, NY 12210. 416 pp. Price: Sw.Fr. 60.-; US$30.00. ISBN 92-8-321229-0.

This volume contains 18 monographs on some industrial chemicals and dyestuffs recently reviewed by an IARC Working Group. The group reviewed the evidence for the carcinogenicity to humans and experimental animals of benzene, benzidine, benzotrichloride, 4,4'-diaminodiphenyl ether, 3,3'-dichlorobenzidine, di(2-ethylhexyl)-phthalate, Direct Black 38, Direct Blue 6, formaldehyde gas, 2-nitropropane, benzyl chloride, benzal chloride, benzene, para-benzoquinone dioxime, di(2-ethylhexyl)-adipate, Direct Brown 95, benzoyl chloride, butyl benzyl phthalate, and ortho- and para-dichlorobenzene. An annex discusses available methods of quantitative risk assessment and the limitations inherent in such assessments, using epidemiological data on benzene and benzidine as examples.

Chromatography: Fundamentals and Applications of Chromatography and Electrophoretic Methods. Part A: Fundamentals and Techniques, Part B: Applications. Edited by Erich Heftmann. Published by Elsevier Scientific Publishing Co., PO Box 330, 1000 AH Amsterdam, The Netherlands, 1983. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017. Part A, 410 pp. Price: US$83.00; Dfl. 195.00. ISBN 0-444-42043-6. Part B, 582 pp. Price: US$138.25; DFl.325.00. ISBN 0-444-42044-4. To order Parts A and B:
ISBN 0-444-42045-2.

An up-to-date treatment of the fields of chromatography and electrophoresis, this 2-volume publication includes changes in techniques since last presented in 1975. Part A gives the theoretical and instrumental basis of each technique. Part B describes the applications of chromatography and electrophoresis to specific classes of compounds.

Mass Spectrometry Advances 1982. Proceedings of the 9th International Mass Spectrometry Conference, Vienna, Austria, 30 August–3 September, 1982. Edited by E. R. Schmid, K. Varmuza, and I. Fogy. Reprinted from the *International Journal of Mass Spectrometry and Ion Physics*, Vols. 45–48. Published by Elsevier Scientific Publishing Co., PO Box 211, 1000 AH Amsterdam, The Netherlands, 1982. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017. 1932 pp. Price: US$361.75; Dfl.850.00. ISBN 0-444-42160-2.

This 2-volume proceedings contains over 2000 papers, including 7 plenary and 20 keynote lectures and 400 contributed papers. These studies contribute to basic scientific knowledge and to solutions of worldwide problems such as the fight against pollution, the improvement of materials and technologies, and the study of chemical, medical, and biochemical processes.

Handling Chemicals Safely. Published by Veiligheidsinstituut, Postbus 5665, 1007 AR Amsterdam, The Netherlands, 1980. Also available from Lab Safety Supply, PO Box 1368, Janesville, WI 53547-1368. 1013 pp. Price: $75.00. ISBN 90701-85-03-02.

This book identifies the hazards for most common chemicals and gives specific instructions for protection, storage, and handling. It includes a safety data profile for 874 chemicals. For each chemical reviewed, the book includes name, synonyms, NFPA values, CAS No., UN No., physical properties, characteristics, fire and explosion hazards, fire fighting, first aid, spillage, storage, and packaging. The manual can serve as a source of information for preparing the labels soon to be required by new OSHA labeling standards and contains most of the information needed for the preparation of Material Safety Data Sheets.

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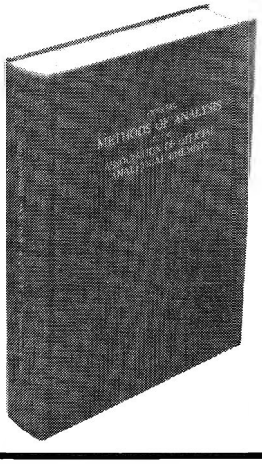

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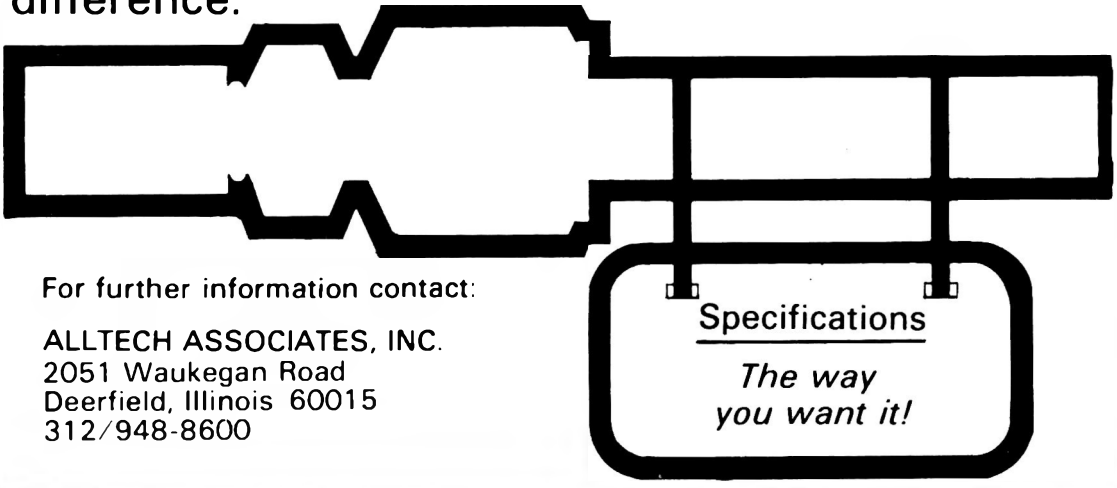

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