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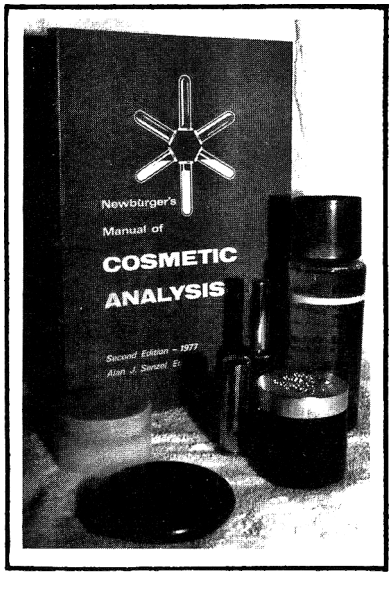

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kg	kilogram(s)
g	gram(s)
mg	milligram(s)
μg	microgram(s)
ng	nanogram(s)
L	liter(s)
mL	milliliter(s)
μL	microliter(s)
m	meter(s)
cm	centimeter(s)
mm	millimeter(s)
μm	micrometer(s) (*not* micron)
nm	nanometer(s) (*not* millimicron)
A	ampere(s)
V	volt(s)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

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

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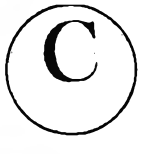

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$^{13}C/^{12}C$ Ratios of Citrus Honeys and Nectars and Their Regulatory Implications

JONATHAN W. WHITE, JR and FRANK A. ROBINSON[1]
Honeytech, Inc., Navasota, TX 77868

Application of isotope ratio testing for adulteration to predominantly citrus honeys without using the recommended confirmatory testing has caused regulatory difficulties for several Florida honey packers. It is demonstrated that $\delta^{13}C$ of citrus honey is significantly less negative than that of other honey types. Analysis of citrus nectar shows that the less negative values are not due to the inadvertent mixing of cane or corn syrups used for spring feeding of the bee colonies previous to the citrus nectar flow. Means for identifying citrus honey and specific limiting values of $\delta^{13}C$ for adulteration testing of this honey type are proposed.

Use of the stable carbon isotope ratio to demonstrate the addition to honey of cane or corn syrups is now well established (1). The average $\delta^{13}C$ value for 119 honey samples (84 United States, 35 imported) was −25.4‰ (s = 0.98‰) and for corn syrup, −9.7‰ ($\delta^{13}C = [(^{13}C/^{12}C$ sample$/^{13}C/^{12}C$ std$) - 1] \times 10^3$). Values for mixtures of honey and corn syrup are the weighted sum of the value for each component. Individual values have been published (2). Although the composition of a mixture can be established within a few percent when the $\delta^{13}C$ value of the honey component is known (ref. 1, Table 5), the variability encountered among the authentic samples precludes accurate calculation of the composition of unknown mixtures. In fact, samples with values between −23.5‰ (2s) and −21.5‰ (4s) should be confirmed as adulterated by an independent test (3). Nonetheless, at least one laboratory has regularly declared samples to be adulterated when such values were obtained, without additional testing. During the 1980 season, this practice caused some marketing difficulty with the Florida citrus honey crop. In one instance a citrus sample with $\delta^{13}C = -23.8$‰ was said to be 10% adulterated, resulting in considerable confusion and economic loss.

Many $\delta^{13}C$ values for citrus honey were encountered that were within the range requiring additional testing; in all cases, the result was negative. It thus seemed advisable to establish the isotope ratio ranges to be expected with authentic citrus honey, with the hypothesis that this floral type differs from other U.S. honeys in this regard. Accordingly, samples of "orange" (citrus) honey from bulk shipments to a Florida packer from long-established producers were analyzed for stable carbon isotope ratio. Results confirmed the less negative nature of citrus honey $\delta^{13}C$ compared with averages for all honey determined in an earlier study. However, the origin of this difference was not resolved because stimulative feeding was a common practice for most producers.

Because the citrus nectar flow comes so early in the year, it is frequently necessary to feed honeybee colonies with honey or sugar syrup to ensure that they are strong enough to take full advantage of the usually heavy nectar flow. This stimulative feeding is a necessary and normal management practice, and beekeepers are careful to avoid mixing stores from such feeding with surplus honey removed for sale. The recent high cost of sugar has encouraged substitution by high-fructose corn syrup for bee feeding. Hence, it is conceivable that the less negative $\delta^{13}C$ values usually found for citrus honey actually represent a consistent, inadvertent admixture of corn or cane syrup used for the bee feeding before the flow.

To resolve this question, isotope ratio measurements were made on a series of citrus nectars collected directly from the blossoms, thus eliminating any possible contamination by other sugars. We found that $\delta^{13}C$ values of citrus nectars are identical with those found earlier for citrus honey, showing that no appreciable admixture with feeding sugars took place under the normal beekeeping practice, and that the dif-

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Table 1. $\delta^{13}C$ values (‰) of honeys and nectars

Sample	No. samples	Mean	Range		SD	CV, %
			High	Low		
Citrus honey[a]	15	−23.8	−22.1	−25.5	0.96	4.03
Citrus nectar	17	−23.3	−22.0	−24.6	0.74	3.17
U.S. honey[b]	84	−25.2	−22.5	−27.4	0.94	3.73

[a] Negative TLC tests were obtained on each of the 5 samples tested (see text).
[b] From ref. 1.

fering $\delta^{13}C$ values of citrus honey are indeed characteristic of that type of honey.

Experimental

Samples

Citrus honey.—Samples were taken from stored bulk Florida citrus honey (1980 crop) as supplied to a Florida packer by 15 producers who regularly sell honey to this packer. Stable carbon isotope ratio was determined for each sample and in addition, the 5 samples with $\delta^{13}C$ values less negative than −23.1‰ were tested for high fructose corn syrup by the charcoal column-TLC method (4).

Citrus nectar.—All nectar samples were collected from mature but unopened citrus buds. By selecting buds whose petals were just beginning to separate, a slight pressure on the tip of the bud would cause the petals to open fully providing easy access to the accumulated nectar, which was collected in 10 or 20 μL capillary pipets. After being filled, the ends of the pipets were flame-sealed, and the sealed tubes were placed in boiling water to sterilize the nectar sample to prevent fermentation.

Determination of $\delta^{13}C$

Stable carbon isotope ratio was determined generally according to method **31.150** (4) by Coastal Science Laboratories, Inc., Port Aransas, TX. Combustion was carried out according to Sofer (5) in a tube in which the sample had been dried. Copper oxide was added, the air was evacuated, the tube was sealed and heated in a furnace, and the CO_2 was purified as specified in **31.150** (4).

Results and Discussion

The results of the analyses of the 1980 citrus honeys and the 1981 citrus nectars are summarized in Table 1, which also shows data for 84 U.S. honeys studied earlier (1). Table 2 shows the individual results for the nectar samples.

Application of the *t*-test showed that the mean values for the citrus honey and for the citrus nectar do not differ significantly (t = 1.66, 30 DF; $t_{0.10}$, 30 DF = 1.697). The generally less negative $\delta^{13}C$ values for citrus honey in comparison with other floral types of honey therefore do not arise from inadvertent contamination with sugar used before the nectar flow for spring build-up of the bee colony, but are characteristic of the plant.

The mean values for citrus honey and all U.S. honey are significantly different (t = 5.11, 97 DF; $t_{0.01}$, 100 DF = 2.64). This implies that use of isotope ratio values for judgment of adulteration of honey known to be of citrus origin should properly be based on mean and variability data

Table 2. $\delta^{13}C$ values of Florida citrus nectars

Variety	Location	Collection date	$\delta^{13}C$, ‰
Orange			
Parson Brown	Clermont	3/19/81	−23.2[a], −22.3, −23.2
Valencia	Clermont	3/19/81	−23.6, −23.2, −22.7
	Lake Placid	3/08/81	−22.4, −23.0
	St. Petersburg	3/23/81	−23.4
Temple	Clermont	3/17/81	−23.3
	Clermont	3/19/81	−24.4
Tangelo	Clermont	3/19/81	−23.3, −22.0, −24.2
	Clermont	3/17/81	−23.6
	Clermont	3/25/81	−24.6
Grapefruit	Clermont	3/30/81	−24.3

[a] Each value represents a single determination on a different sample.

for citrus honey, not all honey in general. The 4s limit proposed earlier (1) for declaring honey to be unquestionably adulterated (−21.5‰) should logically be replaced for citrus honey by −20.0‰. This value was obtained by subtracting 4s (4 times the standard deviation of 0.96‰) from the mean value for citrus honey of −23.8‰ (Table 1). Confirmation by the TLC test should be required for a citrus sample if the value is between −21.9‰ (2s) and −20.0‰ (4s). The use of 4s (assuming a normal distribution for $\delta^{13}C$) means that less than 1 time out of 10 000 would a pure sample falsely be declared adulterated. Such conservatism could lead to frequent acceptance of adulterated samples as unadulterated unless accompanied by the recommended TLC and/or methyl anthranilate testing described below.

Application of these criteria requires that a honey be identifiable as principally citrus in origin. This is not difficult because the flavor and aroma of such honey is quite distinctive and is easily recognized. Furthermore, citrus honeys are unique among U.S. honeys in containing methyl anthranilate (6–8). (Lavender honey, not produced in the United States, it also reported to contain this substance (9)), Knapp (8) has stated that only 1 in 1000 citrus honeys would be expected to contain less than 1.5 ppm. The 80 citrus honeys studied (7, 8) averaged 3.8 ppm (range 0.84–4.9). White (7) reported an apparent methyl anthranilate content averaging 0.07 ppm for 12 non-citrus honeys.

It may reasonably be concluded that Florida citrus honey has significantly less negative $\delta^{13}C$ values than does other U.S. honey, and requires different standards for the determination of adulteration by this measurement. These values are not less negative than those of other honey because of possible contamination with feeding sugar, but are characteristic of the plant species.

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Reverse Phase Liquid Chromatographic Determination of Sulfathiazole Residues in Honey

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Sulfathiazole residues were extracted from honey by homogenizing samples in acetone, filtering, and then evaporating the acetone under nitrogen at 40°C. The remaining extract was transferred to a separatory funnel with 1N HCl and ethyl ether. An aliquot of the retained acid layer was screened by using the Bratton-Marshall reaction. If the test was positive, the remaining portion was analyzed directly through a μBondapak phenyl column monitored by a UV detector at 254 nm. The mobile phase was potassium phosphate monobasic in 10% acetonitrile adjusted to pH 3.0. Time for elution was 13 min. Average recoveries were 78% at the 0.1 ppm spiking level and 68% at the 1.0 ppm level. The minimum detectable amount was 0.06 ppm based on a spiked sample extract.

The antibiotic sodium sulfathiazole is used in the prevention and treatment of American foulbrood in bees. In the United States, federal regulations commonly limit sulfonamide residues to 0.1 ppm in edible tissues (1).

Sulfathiazole residues in various substrates have been determined by many methods. Gas-liquid chromatography with electron capture detection of sulfonamides in edible tissue has been reported by Goodspeed et al. (2), following pH adjustment, extraction, diazomethane methylation, and acylation with pentafluoropropionic anhydride. Daun (3), determining sulfonamides in extracted feeds, used derivatization by diazomethane and heptafluorobutyric anhydride followed by electron capture detection.

Methods have been reported for sulfathiazole residues in honey. Bregha-Morris (4) developed a colorimetric procedure based on the Bratton-Marshall reaction, which could give false positives because color development occurs with sulfonamides in general. The thin layer chromatographic method described by Grandi (5) as sensitive to the 0.1 ppm level involves column cleanup and is not sufficiently quantitative for residue analyses. The high performance liquid chromatographic (HPLC) method proposed by Belliardo (6) is reported to be quantitative to the 1 ppm level but no supportive recovery data are given. Attempts to duplicate this work were unsuccessful. The purpose of the present study is to develop a method to ensure that Canadian honey is free from sodium sulfathiazole residues to the detection limit of 0.06 ppm.

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METHOD

Apparatus

(a) *Liquid chromatograph.*—Altex Model 110A solvent metering pump with flow rate set at 1.1 mL/min (Altex Scientific Inc., Berkeley, CA), equipped with Rheodyne 7125 loop injector (Rheodyne Inc., Berkeley, CA).

(b) *UV detector.*—Spectra-Physics SP 8300 fixed wavelength UV/visible detector with 254 nm filter kit set at sensitivity of 0.0025 absorbance unit full scale (Spectra-Physics, Santa Clara, CA).

(c) *Recorder.*—Linear, with 40 cm/h chart speed (Linear, Irvine, CA).

(d) *Chromatographic column.*—Stainless steel, 30 cm × 3.9 mm id μBondapak phenyl (Waters Associates Inc., Milford, MA).

(e) *Blender.*—Hamilton Beach Model 33.

Reagents

(a) *Acetone.*—Distilled in glass (Caledon Laboratories Ltd, Georgetown, Ontario, Canada).

(b) *Ethyl ether.*—ACS reagent grade (Caledon Laboratories Ltd).

(c) *Acetonitrile.*—HPLC grade (Caledon Laboratories Ltd).

(d) *HPLC mobile phase.*—Dissolve 13.6 g potassium phosphate monobasic in 1 L acetonitrile-water (10 + 90), adjust to pH 3.0 with phosphoric acid, and filter through 0.5 μm polytetrafluoroethylene (Type FH, Millipore Co., Bedford, MA). Degas by using a water aspirator vacuum. Use deionized water.

(e) *Standard solution.*—Dissolve 13 mg sodium sulfathiazole.$1\frac{1}{2}$ H_2O (11.85 mg sodium sulfathiazole) in 1N HCl in 25 mL volumetric flask and dilute to volume with 1N HCl.

(f) *N-(1-Naphthyl)ethylenediamine dihydrochloride (NED).*—Sigma Chemical Co., St. Louis, MO. 0.1% aqueous solution. Refrigerate solution when not in use.

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(g) *Ammonium sulfamate.*—0.5% aqueous solution. Refrigerate solution when not in use.

(h) *Sodium nitrite.*—0.1% aqueous solution. Refrigerate solution when not in use.

Sample Preparation

Weigh 50 g honey into 250 mL Erlenmeyer flask. Add 25 mL acetone and blend 2 min at low speed with Hamilton Beach blender controlled by rheostat. Decant acetone into a 250 mL flask and filter through No. 1 Whatman paper. Repeat acetone extraction twice. Evaporate acetone in 40°C water bath under gentle stream of nitrogen, and quantitatively transfer extract to 250 mL separatory funnel with 20 mL 1N HCl followed by 20 mL ethyl ether. Gently shake separatory funnel and let 2 phases separate. Collect acid phase in graduated cylinder and dilute to 30 mL. Pipet 15 mL aliquot into graduated centrifuge tube.

Bratton-Marshall Screening

To solution in graduated cylinder, add 2 mL sodium nitrite solution, mix, and let stand 3 min. Add 2 mL ammonium sulfamate solution, mix, and let stand 2 min. Add 1 mL NED solution, mix, and let stand 15 min. Sample is positive if red-purple color develops. If B-M test is positive, continue with HPLC confirmation.

Determination

Divide solution in centrifuge tube into equal parts. Spike one part with 5 μL standard solution, which is equivalent to 0.2 ppm sodium sulfathiazole. Determine amount of sodium sulfathiazole present by method of standard additions. Inject 20 μL sample before and after addition of standard. Measure peak heights for sample and spiked sample and determine sodium sulfathiazole as follows:

ppm Sodium sulfathiazole = [(peak height sample)/(peak height spiked sample − peak height sample)] × 0.2 ppm

The minimum detectable amount was 0.06 ppm. This is the minimum concentration of sodium sulfathiazole in a spiked sample extract that results in a signal-to-noise ratio of 2.

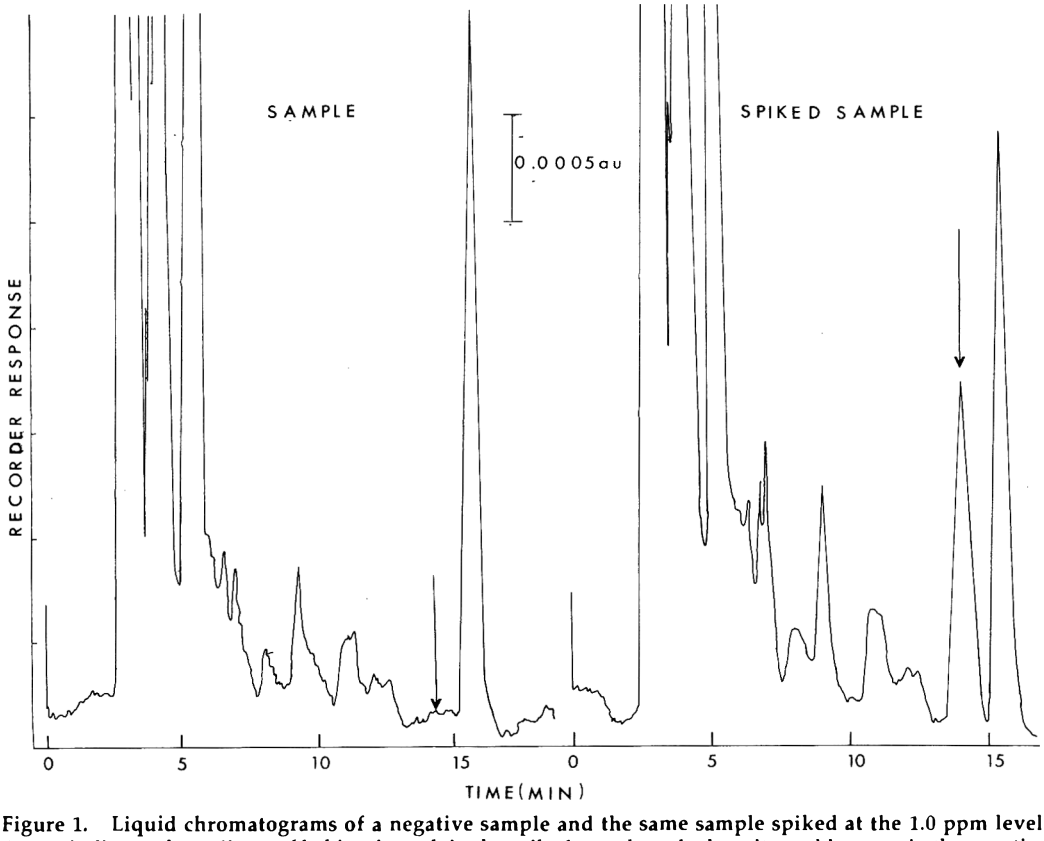

Figure 1. Liquid chromatograms of a negative sample and the same sample spiked at the 1.0 ppm level. Arrow indicates the sodium sulfathiazole peak in the spiked sample and where it would appear in the negative sample.

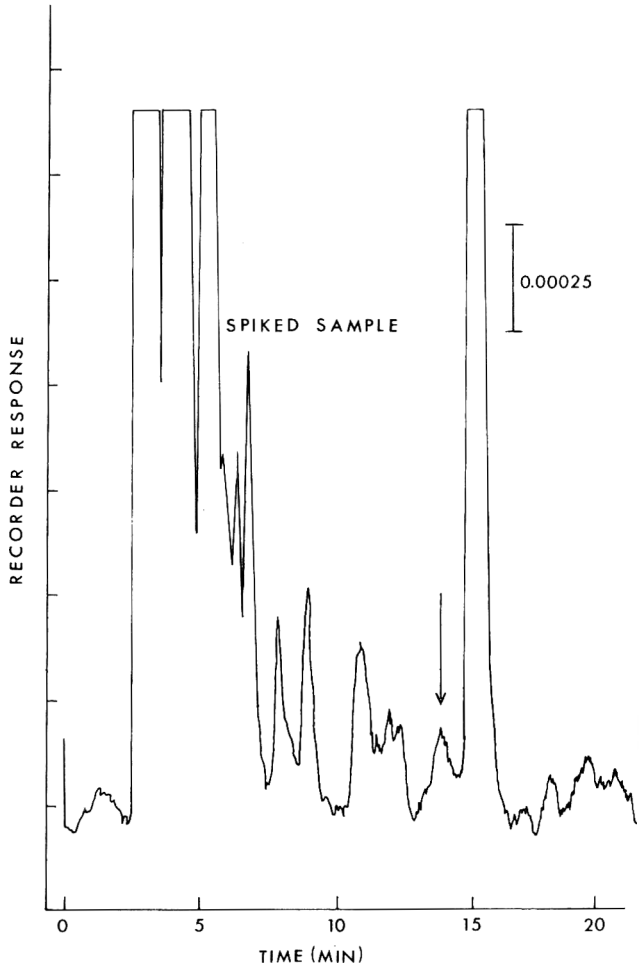

Figure 2. Liquid chromatogram of a blank sample spiked with the minimum detectable quantity, 0.06 ppm. Arrow indicates the sodium sulfathiazole peak.

Results and Discussion

Several sample preparation procedures were tried to obtain optimum extraction efficiency. Acetone proved to be a better extracting solvent than chloroform. Homogenizing the sample before extraction with 5 mL water or 1N HCl did not improve recovery. Homogenizing the sample at low speed rather than centrifuging or shaking was more efficient. The Hamilton Beach blender was capable of homogenizing relatively small amounts of such a viscous substance better than a Waring or Polytron blender.

The pH of the mobile phase was critical to ensure complete ion suppression and separation from interfering compounds. The mobile phase was stable over a period of a day, giving an average retention time of 13.25 ± 0.06 min. The phenyl column resolved the sulfathiazole peak from interferences better than the C_{18} or CN column.

Nineteen commercial honeys collected by Agriculture Canada inspectors were analyzed. The honeys originated from various sources including buckwheat, clover, and natural. Liquid, cream, pasteurized, and nonpasteurized honey were analyzed. Twelve samples were positive by the screening test. Because the B-M reaction forms a color derivative with all primary aromatic amines, HPLC confirmation is necessary. The presence of sulfathiazole residues was confirmed in 8 samples at levels from 0.10 to 0.56 ppm. Chromatograms of a negative sample and a negative sample fortified with 1 ppm sulfathiazole are shown in Figure 1. Figure 2 is a chromatogram of a sample extract spiked with the minimum detectable amount of sulfathiazole.

Table 1. Recovery (%) of sodium sulfathiazole added to honey

Sample	Added, 0.1 ppm	Sample	Added, 1.0 ppm
1	73	1	65
2	70	2	70
3	65	3	83
4	87	4	69
5	95	5	72
		6	51
Av.	78		68.3
SD	12.5		10.4
CV, %	16.1		15.2

To determine the recovery of sodium sulfathiazole, samples of honey were spiked with a solution of sodium sulfathiazole to give added levels of 0.1 and 1.0 ppm, and were analyzed by the present method. Results are shown in Table 1. The variation in recoveries could be due to the differences in the individual honeys as described previously. To study the repeatability of the method, a positive sample was analyzed 3 successive times. Results for the 3 determinations were 0.56, 0.59, and 0.52 ppm sulfathiazole (average, 0.56; SD, 0.04; CV, 7.14%).

Sodium sulfathiazole gave a linear response from 0 to 2.8 ppm. Linear regression of the amount injected vs peak height gave a correlation coefficient of 0.9948.

Preliminary work with an amperometric electrochemical detector connected in series to the system was promising. Eight of the samples were also confirmed electrochemically with an applied voltage of 0.89 V. By controlling the applied voltage, peak heights equal to those of the UV detector could be achieved with a much lower attenuation. Further work with this detector could possibly increase the sensitivity and selectivity of the analysis.

In conclusion, the method presented enables the determination of sodium sulfathiazole residues in honey to the 0.06 ppm level by HPLC.

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TOBACCO

High Performance Liquid Chromatographic Determination of Selected Gas Phase Carbonyls in Tobacco Smoke

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Low molecular weight gas phase carbonyls in tobacco smoke are separated as 2,4-dinitrophenylhydrazones and determined by reverse phase high performance liquid chromatography. A trapping procedure is used whereby the gas phase carbonyls are reacted with the derivatizing reagent in a closed system. The deliveries of acetaldehyde and acrolein are compared with published data. In addition, propionaldehyde and acetone deliveries of selected cigarettes are reported.

Much of the cilia toxicity of tobacco smoke is associated with low molecular weight carbonyls, including acetaldehyde and acrolein (1, 2). These constituents have been determined by the cryothermal trapping of the gas phase of the cigarette smoke followed by transferring the constituents to a gas chromatograph. Various trapping systems have included a copper radiator trap immersed in liquid nitrogen (3), a low temperature still (4), and the cryothermally cooled head of a gas chromatographic (GC) column (5, 6). The latter procedure (6) is currently in use at this laboratory. The gas chromatographic analysis requires about 2 h/cigarette; thus, the number of cigarettes which can be analyzed is limited. Of greater concern is that authentic gas phase standards cannot be conveniently injected into the chromatograph in a quantitative manner. To overcome this problem, a reference cigarette is smoked and analyzed, and carbonyl deliveries are quantitatively determined by ratioing peak areas.

Determination of the carbonyls as their 2,4-dinitrophenylhydrazones (DNPHs) by high performance liquid chromatography (HPLC) (7–14) inherently offers the potential of greater sample throughput as well as increased sensitivity. Also, absolute standardization is possible. Several investigators (7, 8, 15) have used DNPH derivatization for the determination of low molecular weight carbonyls in cigarette smoke by both HPLC and GC. However, few comparisons have been made of the apparent levels of acetaldehyde and acrolein in smoke as determined by this method with those determined by other procedures for reference cigarettes under well defined smoking procedures. In some cases, discrepancies between values obtained by DNPH/HPLC and those of more accepted procedures have been considerable. For example, acetaldehyde and acrolein levels in the smoke of 3 Japanese commercial cigarettes, 2–2.7 mg/cigarette and 460–680 g/cigarette, respectively, (8) were considerably greater than the highest values observed (1.3 mg and 225 μg/cigarette, respectively) when more than 120 varieties of experimental cigarette smokes were assayed as part of the National Cancer Institute Smoking and Health Program (16–19).

In earlier work (20), we reported on a closed trapping/DNPH derivatization/HPLC approach to the measurement of low molecular weight carbonyls in cigarette smoke and other gas phase mixtures. The analytical separation is similar to that published by Fung and Grosjean (21). However, reference cigarette deliveries of these constituents, particularly acrolein, tended to be somewhat lower than accepted values. Here, we detail a modified trapping/analytical procedure for tobacco smoke that provides values which are in good agreement with those determined by accepted gas chromatographic procedures.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Two Waters Associates (Milford, MA) Model 6000A chromatography pumps, a Waters WISP 710A sample processor, a Waters Model 720 system controller and a Waters Model 440 absorbance detector operated at 365 nm. A Waters Model 730 data module is used for data readout.

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(b) *Smoke collection flask.*—Borosilicate glass 1 L flask equipped with 2 side arms: one for evacuation, the other for connection to smoking machine. Each side arm is sealed from atmosphere by means of rubber tubing and pinch clamp. Smoke outlet side arm extends inside flask to within ca 3 mm of bottom of flask.

(c) *Smoking device.*—Single port, Filamatic smoking machine as described and diagrammed by Horton and Guerin (22).

Reagents

(a) *Reference solutions.*—Prepare carbonyl-DNPH derivatives for calibration purposes by conventional procedures (23). Dissolve standards in methanol or chloroform on w/v basis and store refrigerated. Solutions are stable several weeks.

(b) *Trapping solution.*—Prepare 2,4-dinitrophenylhydrazine (DNPH) trapping solution (ca 4 mg/mL) by saturating 200 mL 2M HCl with DNPH (J. T. Baker Chemical Co.).

(c) *Solvents.*—Reagent grade $CHCl_3$ (ACS certified, or equivalent); methanol (distilled in glass, Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

(d) *HPLC elution solvents.*—Water (distilled in glass, Burdick & Jackson Laboratories, Inc.); methanol (distilled in glass). Degas by sonication for 20 min under vacuum.

Procedure

Transfer 40 mL trapping solution, 20 mL chloroform, and Teflon®-coated magnetic stirring bar to borosilicate glass 1 L collection flask. Reduce pressure inside flask via house vacuum or pump to ca 100 torr. Then place flask on magnetic stirrer and attach to single port analytical smoking machine. (Flask is attached to switch S-2 of smoking machine as diagrammed in Figure 2 of Ref. 22.) Condition previously selected cigarettes 48 h at 72–76°F and 60% relative humidity, and smoke under standard conditions (24) of 35 mL, 2 s duration puffs at 1 min intervals through Cambridge filter assembly. Collect smoke gas phase samples by transferring smoke, puff by puff, from one cigarette directly into smoke collection flask containing 2,4-DNPH derivatizing reagent and chloroform extractant. Stir mixture while cigarette is smoked. (In previous work (20), trapping solution was static during collection, and apparent recoveries of acrolein were lower than those reported here.) At end of smoking period, seal off flask by reattaching pinch clamp and remove from smoking machine. After carefully opening inlet side arm to let pressure inside flask equalize, place on shaker 30 min. Following reaction period, transfer contents of flask to separatory funnel and drain chloroform layer (bottom). Extract aqueous phase a second time with 5 mL chloroform. Combine chloroform extracts and wash with 5 mL 2M HCl followed by 10 mL water. Adjust final volume to 25.0 mL. Analyze aliquot of this solution by HPLC.

High Performance Liquid Chromatography

Perform HPLC analysis as follows: analytical column, Dupont Zorbax ODS (4.6 mm × 25 cm), or equivalent, operated at ambient temperature. Mobile phase, methanol–water (70 + 30) at flow rate of 1 mL/min (may vary somewhat to obtain maximum separation efficiency); recorder chart speed, 0.5 cm/min. Set absorbance detector at 0.1 absorbance unit (may vary depending on concentration of samples and standards being analyzed). Normally inject 10 to 20 μL onto LC column.

Perform quantitation by manual measurement of peak height or electronic measurement of peak area.

Calculations

Calculate carbonyl content of smoked cigarettes as follows:

Carbonyl content (mg/cig.)
= (concn std (mg/mL)/area std) × area sample
× final vol. sample (mL)/No. of cigs smoked

Adjust concentration of standard solutions chromatographed to obtain approximately the same area for standards and sample solutions. Use same volume injected onto LC column (usually 10 μL) for standards and samples.

Results and Discussion

It has been shown that low molecular weight carbonyls can be quantitatively trapped by flowing the cigarette smoke through a DNPH trapping solution (7, 8, 15), so this approach was initially examined. That is, after the smoke was passed through a Cambridge filter, the gas phase was diluted with a carrier gas and bubbled through fritted glass in traps containing 2,4-dinitrophenylhydrazine reagent. However, under our conditions (effective flow rate = 17.5 sq. cm/s), the apparent trapping efficiencies of carbonyls from the gas stream were consistently low. For example, apparent acrolein deliveries of reference cigarettes were approximately 40% of published values. Also, the attachment of a second trap in series failed to significantly im-

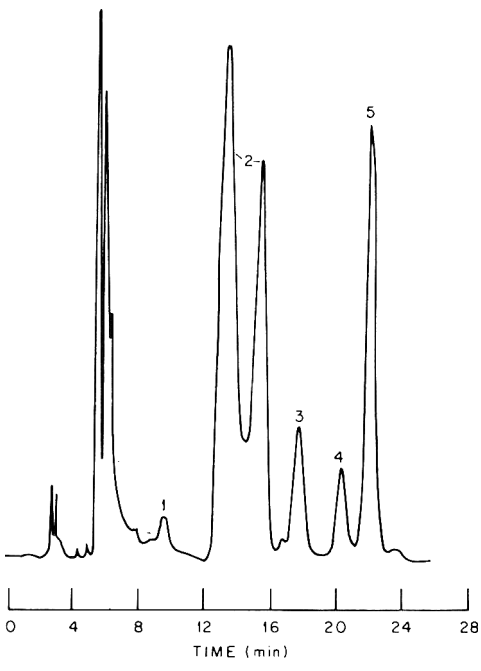

Figure 1. Liquid chromatographic profile of gas phase carbonyl-DNPH derivatives from a Kentucky Reference (1R1) cigarette: (1), formaldehyde; (2), acetaldehyde; (3) acrolein; (4), propionaldehyde; (5), acetone.

prove recoveries. This was true for both standard vapor mixtures and filtered cigarette smoke. Varying the acidity of the trapping solutions and the flow rate of the carrier gas did not significantly improve the results. Under these sampling conditions, it appeared that the residence time of the gas phase in the derivatizing reagent was insufficient for the complete conversion of the carbonyls to 2,4-dinitrophenylhydrazones. Thus, the flow-through system was abandoned in favor of the closed system described above.

A liquid chromatogram of the gas phase carbonyl-DNPH derivatives from the mainstream smoke of a Kentucky Reference 1R1 cigarette is shown in Figure 1. Note that the acetaldehyde–dinitrophenylhydrazone is identified as 2 peaks, not completely resolved. This was observed for both smoke samples and standardization solution, with relative areas of the 2 peaks changing occasionally. Reduction in the amount of the derivative injected onto the HPLC column resulted in no merging of the peaks, suggesting that the phenomenon was not a column overloading effect. Each component of the double peak was subjected individually to high resolution GLC (on a 30 m SE-52 coated fused silica column). The resulting 2 chromatograms each portrayed a pair of peaks, the first and second components of which had respective identical retention times but considerably different peak sizes. The relative size of the 2 peaks was dependent on which of the 2 HPLC fractions was analyzed. High resolution GLC of the combined components of the HPLC peak also yielded 2 peaks with retention times identical to those observed in the chromatogram of the individual components. The mass fragmentation patterns obtained from GC/MS analysis of the HPLC peak for the 2 peaks were identical, and indicative of an acetaldehyde-DNPH derivative. The data suggest that, under these particular HPLC conditions, separation of 2 isomers of the acetaldehyde-DNPH derivative is nearly achieved. The formation of isomeric forms during similar derivatization reactions has been previously re-

Table 1. Comparison of cigarette smoke vapor phase carbonyl deliveries, DNPH/HPLC method vs cryothermal GC

	Deliveries, mg/cig.								
		Acetaldehyde		Acrolein		Propionaldehyde		Acetone	
Cigarette	Tar[a]	HPLC	GC[a]	HPLC	GC[a]	HPLC	GC[b]	HPLC	GC[b]
Kentucky Ref. 1R1[c]	35.6	1.36	1.22	0.11	0.12	0.10	—	0.54	—
RJR (filtered)[d]	17.6	1.14	1.02	0.10	0.11	0.09	—	0.46	—
Code 06[e]	22.3	1.15	0.88	0.22	0.19	0.10	—	0.23	—
Code 21[e,f]	17.1	1.16	0.87	0.06	0.05	0.09	—	0.33	—
Code 23[e]	28.1	1.37	1.02	0.11	0.12	0.11	—	0.39	—
Code 32[e]	25.7	1.28	1.01	0.10	0.11	0.10	—	0.47	—

[a] Ref. 19.
[b] Not determined by GC.
[c] Obtained from University of Kentucky Tobacco and Health Research Institute, Lexington, KY.
[d] Obtained from R. J. Reynolds Tobacco Co., Winston-Salem, NC.
[e] National Cancer Institute Series IV Experimental Cigarettes; see Ref. 19.
[f] Acetaldehyde and acrolein deliveries determined from separate experiment; values not taken from Ref. 19.

Table 2. Gas phase carbonyl deliveries of selected low tar cigarettes

Brand (tar delivery, mg/cig.)	Deliveries, μg/cig.			
	Acetaldehyde	Acrolein	Propionaldehyde	Acetone
A (3 mg)	270	20[a]	20	80
B (2 mg)	90	3[b]	7	20
C (1 mg)	100	3[a]	6	30

[a] Acrolein analysis by GC yielded 19 ± 1 μg/cig.
[b] Acrolein analysis by GC yielded <5 μg/cig.

ported (11, 25). Also of interest, propionaldehyde isomers were not resolved with our system.

Average gas phase deliveries of acetaldehyde, acrolein, propionaldehyde, and acetone from several experimental cigarettes are reported in Table 1. For comparison, values for acetaldehyde and acrolein determined by GC are reported (19). In addition, "tar" deliveries for the cigarettes are reported (19). The relative standard deviation for the measurement of the reported gas phase carbonyls by the HPLC method was about 12%. Good agreement with the GC values was obtained for the acrolein deliveries. The HPLC method yielded somewhat higher values for acetaldehyde than did the GC procedure. The acetone deliveries varied over a wider range than did those of the propionaldehyde. With either the HPLC or GC values, there seemed to be no distinct relationship between tar and acetaldehyde or acrolein levels. To our knowledge, there exists no published data for the mainstream smoke deliveries of propionaldehyde and acetone for these cigarettes. Determination of the gas phase deliveries of formaldehyde was not performed because of the poorly defined formaldehyde-DNPH peaks. However, qualitative relative ranking of the deliveries could be made from visual inspection of the chromatograms.

Because of their ultra-low smoke constituent deliveries, certain low tar cigarettes now being marketed have not been amenable to gas phase acrolein determinations by our gas-liquid chromatographic procedure. To demonstrate the utility of the derivatization/HPLC approach for the determination of gas phase carbonyls from low tar cigarettes, the smokes of 3 low tar brands were analyzed. In this case, the gas phase portions from several cigarettes were collected and analyzed as one. This was accomplished by evacuating the flask, collecting the gas phase, reacting to derivatize the carbonyls, then re-evacuating the flask, and repeating the procedure. Table 2 lists the gas phase carbonyl contents of the 3 brands. For the one brand (brand A) on which acrolein analysis had been possible by conventional means, good agreement between the GC and HPLC determined values was achieved.

The lower limit of detection of carbonyls, for example acrolein, is about 10 μg/cigarette, based on smoking one cigarette and adjusting the final volume of the chloroform extract to 25 mL. Although the volume of chloroform extract could be reduced somewhat by evaporation under a stream of nitrogen, large volume reductions would require additional cleanup of the chloroform because it contains traces of acetaldehyde and acetone.

The best resolution of the propionaldehyde and acetone derivatives was obtained on a relatively new DuPont Zorbax column. On aged columns, the resolution of these 2 peaks deteriorated, with the acetone derivative being the predominant peak. However, resolution of the acetaldehyde and acrolein derivatives continued to be adequate.

Attractive advantages of the liquid chromatographic procedure employing a closed trapping system are that standard samples can be rerun periodically, multiple analyses can be made on the same cigarette, and a greater sample throughput can be achieved. DNPH derivatization followed by HPLC appears to be a reliable and sensitive method for determining acetaldehyde, acrolein, propionaldehyde, and acetone in tobacco smoke. The method should also be applicable to other gas phase matrices.

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INDUSTRIAL CHEMICALS

Selected Ion Mass Spectrometric Identification of Chlorophenol Residues in Human Urine

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A method is presented for the detection and confirmation of trace chlorophenol residues in environmental and biological samples by mass spectrometry with selected ion monitoring (SIM). Propionate and acetate ester derivatives of phenol, cresols, mono-, di-, tri-, and tetrachlorophenols, pentachlorophenol, as well as the internal standard 4,6-dibromo-o-cresol, were prepared directly in aqueous solution, using the appropriate anhydride reagent. The acetate or propionate esters were quantitatively extracted and then separated by gas-liquid chromatography using either an SP-1240DA or OV-101 packed column. Electron impact SIM was carried out by simultaneously monitoring up to 4 diagnostic fragment ions of each chlorinated phenol. The use of SIM eliminated background interference encountered using flame ionization or electron capture detectors, and allowed identification of chlorophenol residues in extracts of urine samples collected from individuals without known exposure to chlorophenols. Phenol concentrations as low as 1.0 pmol/mL urine gave peaks that were readily discernible by SIM.

The widespread use of chlorinated phenols (1, 2) combined with their toxicity (2, 3) and persistence in the environment (4) has generated considerable interest in the development of sensitive, rapid, and reliable methods for their routine analysis. Various analytical procedures exist for quantitation of chlorinated phenols in biological fluids (5–7), tissues (8, 9), river water (10), industrial wastes (11), and many other sample matrices (12). Chlorinated phenols have been analyzed without derivatization by using high performance liquid chromatography (HPLC) (5, 13), negative chemical ionization mass spectrometry (9), and gas-liquid chromatography (GLC) on bonded or specially treated stationary phases (7, 14). Although these direct methods of phenol analysis have been reported, GLC techniques generally involve an initial solvent extraction followed by derivatization and analysis using a flame ionization detector (FID) or an electron capture detector (ECD). Most commonly, ethers prepared with diazoalkane reagents (6, 15) or acetate esters prepared with acetic anhydride (10) are used for identification and quantitation of chlorophenols. A major limitation of these analytical techniques is the high degree of uncertainty associated with the identification of compounds based simply on the coincidence of GLC retention times with those of authentic standards. Quantitation of chlorophenols at parts per billion levels has been reported frequently; however, natural constituents in a biological extract may have GLC retention times similar to the chlorophenols of interest. The reliability of trace analysis using ECD has recently been questioned (2, 4, 5). The EC detector is highly sensitive but it is nonspecific, and additional evidence is necessary to confirm the identities of GLC peaks. Lores et al. (5) advocated the use of HPLC with electrochemical detection for the confirmation of chlorophenols detected in urine by using GLC with ECD.

In the present study, the mass spectrometer (MS) is used as a very sensitive and specific detector in selected ion monitoring (SIM) mode for unequivocal identification of chlorophenol residues. Both propionate and acetate derivatives of phenol (PHE), *o*-cresol (*o*-CRE), *p*-cresol (*p*-CRE), 2-chlorophenol (2-MCP), 4-chlorophenol (4-MCP), 2,4-dichlorophenol (2,4-DCP), 2,6-dichlorophenol (2,6-DCP), 2,4,5-trichlorophenol (2,4,5-TCP), 2,4,6-trichlorophenol (2,4,6-TCP), 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP), pentachlorophenol (PCP), and the internal standard 4,6-dibromo-*o*-cresol (4,6-DBC) are prepared directly in aqueous solution (16–19), using the appropriate reagent anhydride. The derivatives are resolved by gas chromatography and detected by monitoring one or more characteristic fragment ions for each compound by SIM-MS. The sensitivity of SIM compares favorably to sensitivities achieved using FID or ECD, and background interferences are eliminated. Urine samples collected from individuals

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without known exposure to chlorophenols were examined using the developed SIM procedure.

METHOD

Reagents and Samples

(a) *Solvent.*—Methylene chloride, glass-distilled before use.

(b) *Water.*—Deionized, glass-distilled (Corning AG-11, Corning Glassworks, Corning, NY).

(c) *Sodium sulfate.*—Anhydrous, granular, reagent grade. Heat in muffle furnace at 400°C and store in capped (aluminum foil-lined) amber bottle.

(d) *Standard phenol solutions.*—Prepare individual 1 mM solutions of PHE, *o*-CRE, *p*-CRE, 2-MCP, 4-MCP, 2,6-DCP, 2,4-DCP, 2,6-DCP, 2,4,5-TCP, 2,4,6-TCP, 2,3,4,5-TeCP, 2,3,4,6-TeCP, PCP, and 4,6-DBC in 95% ethanol and store in glass-stopper bottles at 4°C. Dilute stock solutions with water for use.

(e) *Urine samples.*—First void samples were obtained from persons in the general population without known occupational exposure to industrial chemicals or pesticides.

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5730A equipped with FID, 15 mCi ^{63}Ni linear ECD, and Model 3380A integrator. Operating temperatures: injector, 250°C, FID, 250°C, ECD, 250°C.

(b) *Combined GLC-MS system.*—Hewlett-Packard Model 5710A gas chromatograph/Model 5981A quadrupole mass spectrometer, with Hewlett-Packard Model 5934A data system for carrying out total ion scanning (40–400 amu) and selected ion monitoring. MS scan conditions: scan speed, 100 amu/s; band width, 430 Hz; electron energy, 10–70 eV; dwell time, 200 ms; ion source temperature, 180°C. Separator temperature same as for oven.

(c) *Chromatographic columns.*—Column 1: 1.26 m × 4 mm id glass packed with 1% SP-1240DA on 100–120 mesh Supelcoport. Column 2: 1.26 m × 4 mm id glass packed with 5% OV-101 on 80–100 mesh Chromosorb W. Operating temperatures: column 1, 75–170°C at 8°/min; column 2, 75–220°C at 8°/min.

Preparation of Samples and Standards

Pipet 5 mL urine or standard (distilled water containing 0.005–1.0 nmole of each phenol listed in *Reagents and Samples* (d)) into a 20 × 125 mm screw-cap (Teflon-lined) test tube and acidify with 0.2 mL concentrated H_2SO_4. To hydrolyze conjugates in the urine samples, place sealed tube in boiling water bath for 2 h. Cool sample to room temperature, add 0.2 mL 1 μM 4,6-DBC internal standard, make basic to pH > 12 with NaOH pellets, and extract with 1 mL methylene chloride to remove basic and neutral organic compounds. Discard organic layer and neutralize partially purified sample with concentrated H_2SO_4 before derivatization. Prepare derivatives by adding 0.5 g $NaHCO_3$ followed by addition of 250 μL of either acetic anhydride or propionic anhydride to aqueous sample. After carbon dioxide evolution ceases, extract acyl derivatives by shaking samples twice for 2 min with 2 mL methylene chloride. (Quantitative recovery of simple phenols by using this procedure has been demonstrated previously (16).) After combining extracts, remove traces of water in methylene chloride by passing solvent through Pasteur pipet containing glass wool plug and ca 1 g anhydrous Na_2SO_4. Rinse Na_2SO_4 column with additional 0.5 mL methylene chloride, and evaporate combined extracts to 100 μL with gentle stream of nitrogen.

Mass Spectral Data

The acetate and propionate derivatives of the phenols were positively identified by GLC-MS. Mass spectral data consistent with the assigned structures of the acyl derivatives of PHE, *o*-CRE, *p*-CRE, 2-MCP, 4-MCP, 2,4-DCP, 2,6-DCP, 2,4,6-TCP, 2,4,5-TCP, 2,3,4,6-TeCP, 2,3,4,5-TeCP, PCP, and internal standard 4,6-DBC are summarized in Table 1.

Discussion

Phenolic hydroxyl groups can be derivatized readily in aqueous alkaline solution by reaction with chloroformate (20) or anhydride (16–18) reagents. In our previous studies (16–18), acetic anhydride was used successfully to prepare acetate derivatives of trace phenols directly in large volumes of water; the excellent GLC characteristics and stability of acyl derivatives of many phenols has already been established. In the present study, direct acylation of an alkaline ($NaHCO_3$) aqueous solution by using acetic anhydride or propionic anhydride completely converted the trace amounts of added PHE, *o*-CRE, *p*-CRE, 2-MCP, 4-MCP, 2,4-DCP, 2,6-DCP, 2,4,6-TCP, 2,4,5-TCP, 2,3,4,5-TeCP, 2,3,4,6-TeCP, PCP, and internal standard 4,6-DBC to their respective acyl derivatives. Using either anhydride, the reactions proceeded rapidly to completion at room temperature and the ester de-

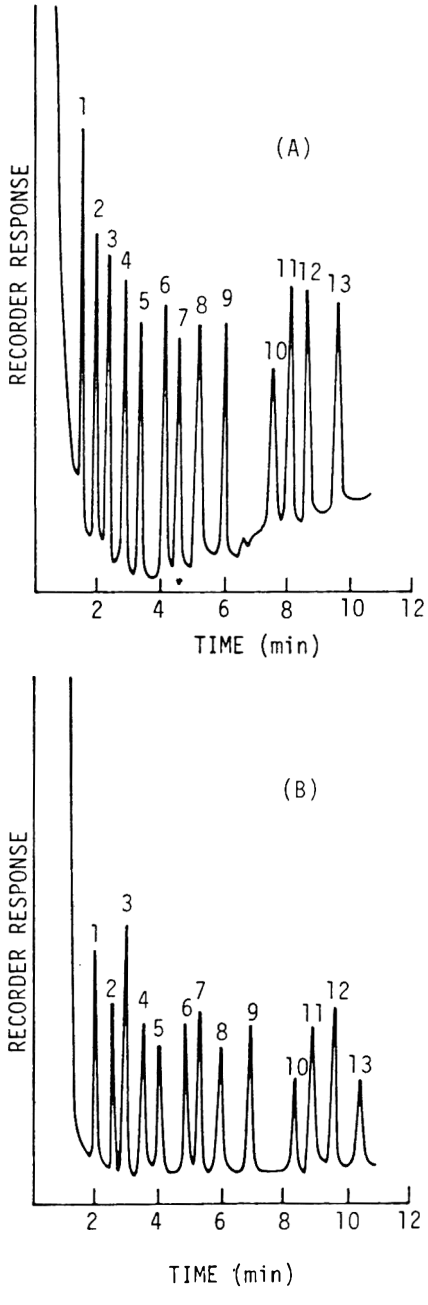


Figure 1. Gas chromatograms obtained by derivatization of 20 nmoles of each phenol in 100 mL water with acetic anhydride (A) and propionic anhydride (B). Chromatographic conditions: 1% SP-1240DA, 75-170°C at 8°/min. Peak identification: 1, phenol; 2, o-cresol; 3, *p*-cresol; 4, 2-chlorophenol; 5, 4-chlorophenol; 6, 2,6-dichlorophenol; 7, 2,4-dichlorophenol; 8, 2,4,6-trichlorophenol; 9, 2,4,5-trichlorophenol; 10, 2,3,4,6-tetrachlorophenol; 11, 4,6-dibromo-*o*-cresol; 12, 2,3,4,5-tetrachlorophenol; 13, pentachlorophenol.

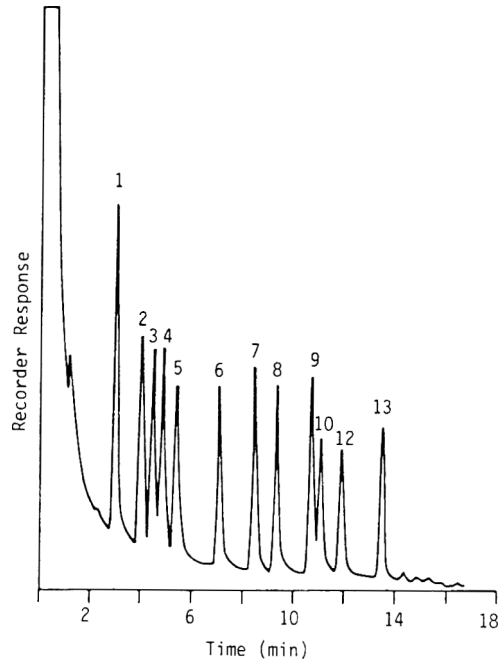
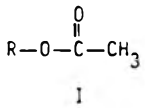


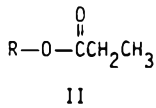
Figure 2. Gas chromatogram of acetate derivatives of chlorophenols resolved on a packed 5% OV-101 column. Chromatographic conditions: 75-220°C at 8°/min. See Figure 1 for peak identification.

rivatives could be efficiently extracted from aqueous solution into methylene chloride. The solvent volumes were reduced under a gentle stream of nitrogen; evaporation of sample extracts to dryness was avoided because erratic results were obtained due to the loss of volatile acyl derivatives. Following this simple one-step acylation procedure, the 13 phenols included in this study were easily separated by using either an SP-1240 or an OV-101 packed column. Typical chromatograms of the acetates and propionates are shown in Figure 1; complete resolution was achieved within less than 12 min. SP-1240DA, a phosphoric acid deactivated polyester material, is thermally unstable above 180°C and is neutralized by basic materials which cause rapid column deterioration (14). SP-1240DA would not be a suitable stationary phase for the analysis of samples containing basic compounds or impurities which could only be eliminated from the column by high temperature baking. OV-101 can be used as an alternative column packing for the GLC analysis of the acylated phenols. Figure 2 is a typical chromatogram of acetate ester derivatives of 12 phenols and the internal standard obtained using an OV-101 column.

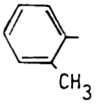
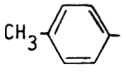
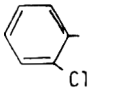
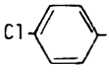
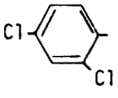
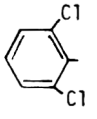
Table 1. Diagnostic fragment ions in the mass spectra (70 ev ion source) of acetate (I) and propionate (II) derivatives



I

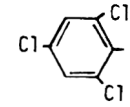
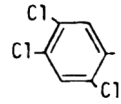
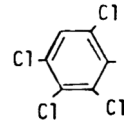
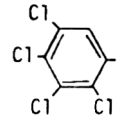
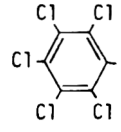
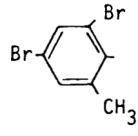


II

Compound	R	m/z (% Relative Abundance)
phenol		I 136[M$^+$](12), 94(100), 43(6) II 150[M$^+$](15), 95(7.7), 94(100), 57(21)
o-cresol		I 150[M$^+$](21), 108(100), 107(46), 43(21) II 164[M$^+$](16), 109(10), 108(100), 107(25), 57(87)
p-cresol		I 150[M$^+$](12), 108(100), 107(57), 43(13) II 164[M$^+$](9), 109(8), 108(100), 107(31), 57(18)
2-chlorophenol		I 170[M$^+$](9), 130(31), 128(100), 43(13) II 186[M$^+$](8), 184[M$^+$](20), 130(33), 129(10), 128(100), 57(89)
4-chlorophenol		I 170[M$^+$](10), 130(34), 128(100), 43(10) II 186[M$^+$](6), 184[M$^+$](16), 130(32), 128(100), 57(45)
2,4-dichlorophenol		I 206[M$^+$](11), 204[M$^+$](18), 164(60), 162(100), 43(18) II 220[M$^+$](8), 218[M$^+$](10), 166(5), 164(18), 162(28), 57(100)
2,6-dichlorophenol		I 206[M$^+$](7), 204[M$^+$](11), 164(63), 162(100), 43(13) II 220[M$^+$](9), 218[M$^+$](11), 166(9), 164(40), 162(63), 57(100)

The identities of the compounds from each peak in Figures 1 and 2 were confirmed by combined GLC–MS. Mass spectral data on the phenols as acetate and propionate derivatives are summarized in Table 1. The mass spectra of the acetates all contained the ions M^+, $(M - 42)^+$, and $(CH_3CO)^+$; the propionate derivatives similarly contained the ions M^+, $(M - 56)^+$, and $(CH_3CH_2CO)^+$. Typical isotopic ion clusters were observed in the spectra of chlorophenols. Characteristic and abundant ions were chosen from the MS data shown in Table 1 for SIM analysis of the 13 phenols as acetate and propionate derivatives. The Hewlett-Packard Model 5934A MS data system is capable of scanning 20 selected masses during a single run. A group of 4 masses was monitored at any one time and a total of 5 groups was selected for each SIM analysis. The ions chosen for SIM analysis of each phenyl acetate and phenyl propionate are shown in Tables 2 and 3, respectively, and the SIM profiles obtained for standard mixtures of the 13 phenols as acetate and propionate esters are shown in Figures 3 and 4, respectively. The standard profiles were prepared by derivatizing 1 nmole of each phenol in 5 mL water. In addition to the ions monitored in Figures 3 and 4, other ions from Table 1 could also be used for SIM. The abundance of the ions scanned could be optimized by changing the ion source voltage.

Table 1. (continued)

Compound	R	m/z (% Relative Abundance)
2,4,6-trichlorophenol		I 242[M⁺](4), 240[M⁺](14), 238[M⁺](14), 200(30), 198(100), 196(97), 43(25) II 256[M⁺](9), 254[M⁺](31), 252[M⁺](31), 200(30), 198(92), 196(100), 57(84)
2,4,5-trichlorophenol		I 242[M⁺](3), 240[M⁺](12), 238[M⁺](12), 200(33), 198(99), 196(100), 43(28) II 256[M⁺] (6), 254[M⁺](17), 252[M⁺] (17), 200(31), 198(98), 196(100), 57(38)
2,3,4,6-tetrachlorophenol		I 276[M⁺](5), 274[M⁺](12), 272[M⁺](9), 234(47), 232(100), 230(77), 43(25) II 290[M⁺](16), 288[M⁺](30), 286[M⁺] (25), 234(49), 232(90), 230(72), 57(100)
2,3,4,5-tetrachlorophenol		I 276[M⁺](4), 274[M⁺](10), 234(49), 232(100), 230(82), 43(26) II 290[M⁺](5), 288[M⁺](12), 286[M⁺] (9), 234(33), 232(65), 230(59), 57(100)
pentachlorophenol		I 312[M⁺](3), 310[M⁺](12), 308[M⁺] (17), 306[M⁺](11), 270(21), 268(59), 266(100), 264(67), 167(22), 165(22), 43(20) II 324[M⁺](12), 322[M⁺](18), 320[M⁺] (12), 270(7), 268(25), 266(38), 264(24), 169(12), 167(38), 165(38), 57(100)
4,6-dibromo-o-cresol		I 310[M⁺](5), 308[M⁺](10), 306[M⁺] (5), 268(52), 266(100), 264(54), 187(25), 185(25), 43(20) II 322[M⁺](10), 320[M⁺](7), 268(38), 266(80), 264(41), 187(18), 185(19), 57(100)

As shown in Figure 5, the relative abundance of fragment ions in the mass spectrum of 2,4-dichlorophenyl propionate was affected by changes in ion source voltage. As the voltage decreased fewer low molecular weight fragments were produced and the abundance of the molecular ion simultaneously increased. In this study, an ionization voltage of 70 eV achieved optimum sensitivity for the detection of the ions chosen for screening and identified in Figures 3 and 4.

The SIM programs shown in Tables 2 and 3 were applied to the determination of chlorophenol residues in acylated extracts of hydrolyzed urine samples. Normal urine samples contain complex mixtures of natural organic compounds which are not removed by the simple extraction–cleanup procedure described in this study. It was impossible to confirm the presence of trace chlorophenols by using FID or ECD because of interferences from organic background. A number of methods for GLC analysis include extensive purification steps on alumina, Florisil, and XAD columns (6–8). Edgerton and Moseman (6) found that column cleanup was essential for the determination of PCP at levels below 30 ppb. Following the analysis of over 400 urine samples collected from the general U.S. population (21), mean PCP and 2,4,5-TCP concentrations were less than 6 ppb.

Table 2. Diagnostic ions used for selected ion monitoring-mass spectrometry of phenols derivatized with acetic anhydride

Peak No.	Acetate derivative of	Group	Ion (m/z)
1	phenol	I	94
2	o-cresol		108
3	*p*-cresol		108
4	2-chlorophenol		128, 130
5	4-chlorophenol		128, 130
6	2,6-dichlorophenol	II	162, 164, 204, 206
7	2,4-dichlorophenol		162, 164, 204, 206
8	2,4,6-trichlorophenol	III	196, 198, 200, 240
9	2,4,5-trichlorophenol		196, 198, 200, 240
10	2,3,4,6-tetrachlorophenol	IV	230, 232
11	4,6-dibromo-*o*-cresol		264, 266
12	2,3,4,5-tetrachlorophenol		230, 232
13	pentachlorophenol	V	264, 266, 268, 270

These concentration levels were at the limits of the specified detection range for the ECD-GLC method used. At these levels, therefore, the phenolic compounds of interest are difficult to distinguish from background or baseline drift, using FID or ECD, even following lengthy isolation procedures. GLC retention times cannot be used for the unequivocal identification of chlorophenols because other components in the sample may possess coincident retention times. SIM-MS provides more selective detection with sensitivity comparable to that for ECD, and can be used to confirm the presence of trace phenol levels in biological extracts.

The detection limit (signal:noise, 2:1) of the chlorophenols identified in Table 1 was 1 pmole/mL when they were added to urine samples and detected by SIM-MS following aqueous acylation. PCP acetate, for example, was detected by adjusting the MS to monitor only the ion currents of the m/z 266 base peak (due to the loss of ketene from the molecular ion) and its isotope cluster ions m/z 264, 268, and 270. As shown in Figure 6, the MS in total ion current mode (B) produced a response for all the volatile organic components in the acetylated urine extract which entered the ion source. Acetylated PCP, present in the sample at a retention time of 9.6 min, was masked by other constituents. In contrast, using SIM (Figure 6A), a detector response was obtained only for the PCP derivative present in the injected sample. Other compounds in the GLC effluent with similar retention times but which lacked the ions being monitored were no longer detected. PCP was unequivocally identified, because all 4 ion current peaks were present at the GLC retention time which matched that of the authentic standard.

The SIM profile of a urine sample derivatized with acetic anhydride is shown in Figure 7. The sample, containing added 4,6-DBC internal standard, contained PHE, *p*-CRE, 2,3,4,6-TeCP, and PCP. To provide conclusive identification of the presence of a phenolic compound, peaks must be observed at all the monitored diagnostic ions at the expected retention time of the standard compound. Although a number of peaks are present in Figure 7, in the ion current groups II and III, DCPs and TCPs were not present in the

Table 3. Diagnostic ions used for selected ion monitoring-mass spectrometry of phenols derivatized with propionic anhydride

Peak No.	Propionate derivative of	Group	Ion (m/z)
4	2-chlorophenol	I	128, 130, 184, 186
5	4-chlorophenol		128, 130, 184, 186
6	2,6-dichlorophenol	II	162, 164, 218, 220
7	2,4-dichlorophenol		162, 164, 218, 220
8	2,4,6-trichlorophenol	III	196, 198, 252, 254
9	2,4,5-trichlorophenol		196, 198, 252, 254
10	2,3,4,6-tetrachlorophenol	IV	230, 232, 288
11	4,6-dibromo-*o*-cresol		266
12	2,3,4,5-tetrachlorophenol		230, 232, 288
13	pentachlorophenol	V	264, 266, 268, 322

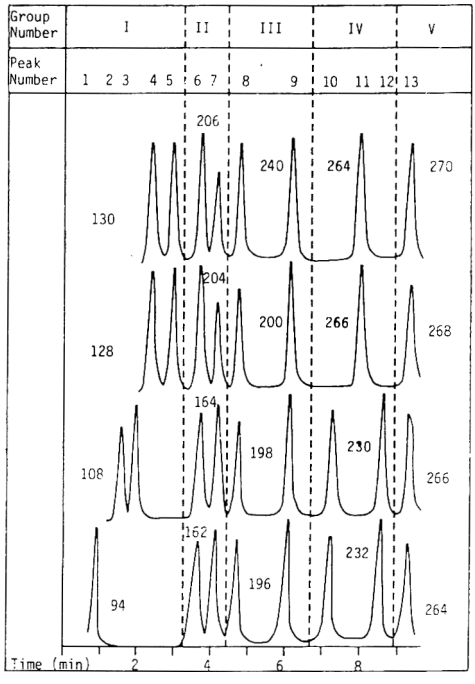


Figure 3. SIM-MS chromatogram of acetate derivatives of a standard mixture of 13 phenolic compounds. Ion groups and phenols corresponding to each peak number are identified in Table 2. Chromatographic conditions: 1% SP-1240DA, 75-170°C at 8°/min. MS scan conditions: electron energy, 70 eV.

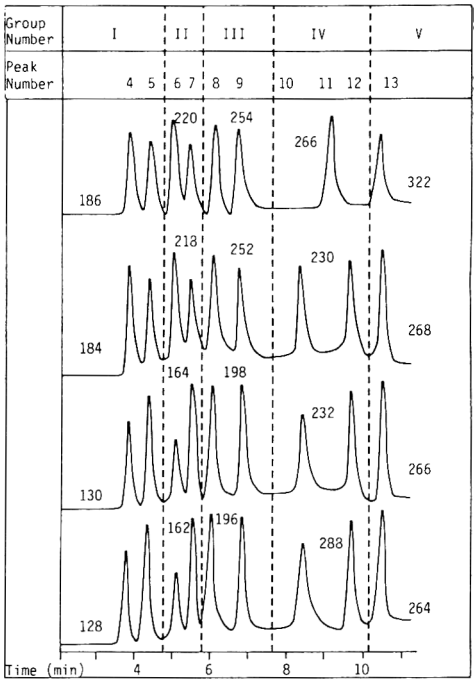


Figure 4. SIM-MS chromatogram of propionate derivatives of a standard mixture of 10 phenolic compounds. Ion groups and phenols corresponding to each peak number are identified in Table 3. Chromatographic conditions: 1% SP-1240DA, 75-170°C at 8°/min. MS scan conditions: electron energy, 70 eV.

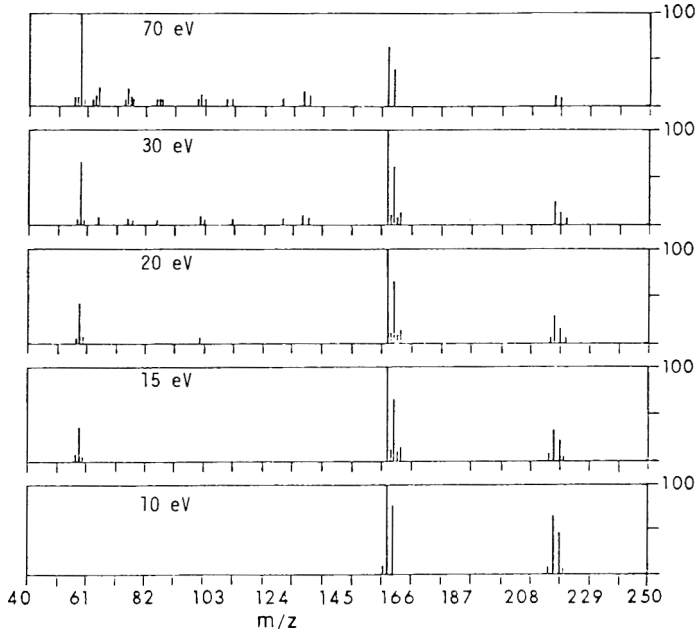


Figure 5. Effect of ion source voltage (10-70 eV) on abundance of fragment ions in the electron impact mass spectrum of 2,4-dichlorophenyl propionate.

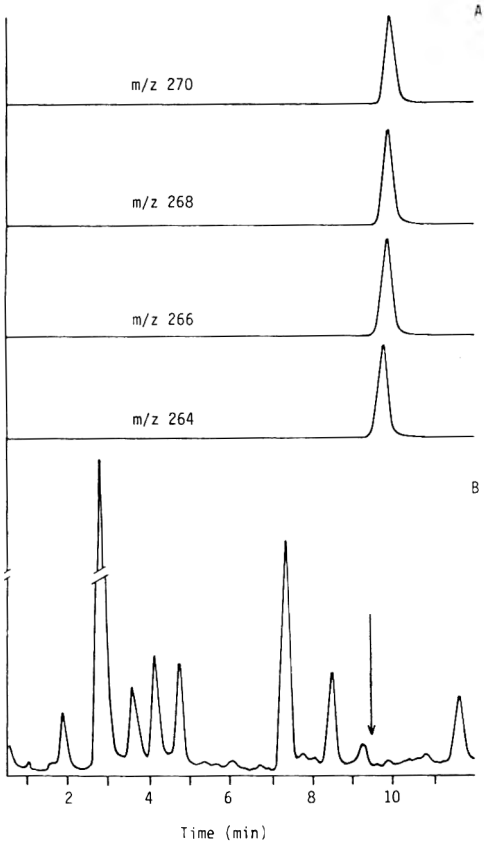


Figure 6. Using SIM-MS (A), derivatized PCP residue in acetylated urine sample is easily distinguished from other natural urinary constituents detected by mass spectrometer in total ion mode (B). Chromatographic conditions: 1% SP-1240DA, 75-170°C at 8°/min.

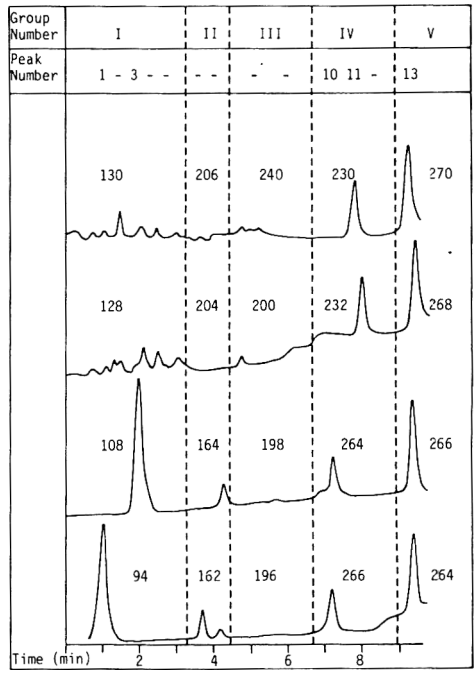


Figure 7. SIM-MS analysis of acetylated urine sample identified presence of phenol (1); *p*-cresol (3); 2,3,4,6-tetrachlorophenol (10); and pentachlorophenol (13). 4,6-Dibromo-*o*-cresol (11) was added as internal standard. Ion groups and peak numbers correspond to SIM-MS program outlined in Table 2. Chromatographic conditions: 1% SP-1240DA, 75-170°C at 8°/min.

sample. To confirm the presence of these chlorophenols, peaks must be observed simultaneously at all of the ion currents set to detect them. Of the 25 urine samples analyzed by SIM-MS following derivatization with both propionic and acetic anhyrides, more than 90% contained detectable levels of PCP.

At very low chlorophenol concentrations, column adsorption phenomena affected the detection of PCP. After many injections onto the SP-1240DA column, PCP could no longer be detected in extracts of samples spiked with the standard mixture of 13 phenols. Wu et al. (22) reported similar effects for analysis of PCP in extracts of rainbow trout tissue. We observed in the present procedure that PCP could no longer be detected if the column became contaminated with extraneous sample constituents. Adsorption effects of the other 12 phenols included in this study were not as pronounced as those experienced with PCP. If the glass wool plug and the first few inches of column packing material were replaced regularly, losses of PCP on-column were minimized.

Quantitative SIM analysis of phenols, using 4,6-DBC as an internal standard, was not possible. The linear relationship between concentration and ion peak area was inconsistent at low concentration levels. Generally, the fragmentation and chemical characteristics of a suitable reference compound for SIM quantitation must closely parallel those of the compound of interest. Bose and Fujiwara (23) prepared PCP benzoate derivatives and used the corresponding pentadeuterated benzoate of PCP as the internal standard for quantitative SIM analysis. Ingram et al. (24) described a mass spectrometric isotope dilution method using ^{18}O-labeled PCP as the reference compound. Wu et al. (22) used pentachlorophenetole as an internal standard for PCP analysis, and a plot of peak height vs concentration was linear. For quantitative analysis,

internal standards other than stable isotope-labeled analogs can be used but results are usually less accurate at low concentrations due to differences in partition coefficient and chromatographic properties of the compound and internal standard. 4,6-DBC, therefore, could be used as a retention-time marker, but was not suitable for quantitative analysis.

SIM-MS can be used as an alternative method for the identification of chlorophenol residues in biological or environmental samples containing interferences which complicate the detection of phenols by FID or ECD. The propionate and acetate derivatives have different retention times under identical GLC conditions. The application of both SIM programs to the analysis of unknown samples can be used as an alternative method for the analysis of biological or environmental samples containing interferences which complicate the detection of phenols by FID or ECD.

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Interlaboratory Calibration Results of Polychlorinated Biphenyl Analyses in Herring

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Twenty-three laboratories from 13 countries in continental Europe, Scandinavia, the British Isles, and North America participated in a polychlorinated biphenyl (PCB) Check Sample Program conducted under the auspices of the International Council for Exploration of the Sea (ICES). PCBs were determined in unspiked and spiked (1.00 mg Aroclor 1254/kg oil) herring (*Clupea harengus harengus*) oil by the participants, each of which used his or her own cleanup and quantitation techniques; a common Aroclor 1254 mixture was used as a standard and a common quantitation technique was used for comparative purposes. Results for the unspiked oil ranged from 0.48 to 3.416 mg PCB/kg oil, while spiked oil results ranged from 0.70 to 3.891 mg/kg. Calculated spike recoveries ranged from 22 to 136%. Serious deficiencies were found in most steps in the procedures. No evidence was found to support the use of a common PCB standard or a common method of calculation using packed column chromatography. The chromatographic stationary phase used appeared to affect the PCB levels obtained. Florisil cleanup adsorbent yielded higher mean results for both unspiked and spiked oils than did alumina. Large coefficients of variation were found (25-50%), the principal source of which was systematic error (interlaboratory).

A number of interlaboratory calibration studies of polychlorinated biphenyls (PCBs) dealing with various components of the marine environment have appeared in the literature in recent years (1-3). Large coefficients of variation (25-30%) have been associated with the results of these studies. Reasons for these large coefficients of variation were not apparent in these earlier studies, for example, the effects of different analytical methodologies were not studied nor were the separate parts of the analytical procedures individually scrutinized for their contributions to the variance. In an attempt to identify the sources of variance, the International Council for the Exploration of the Sea (ICES) Marine Chemistry Working Group agreed to carry out another PCB intercalibration study involving a marine oil and the same oil spiked with a polychlorinated biphenyl mixture. The 23 participating laboratories were allowed complete freedom as to methodology used, but, in addition, were requested to supply results based on a common PCB preparation and a common quantitation method, both supplied by the coordinating laboratory. This report describes the results obtained; the sources of error are also discussed, which have not been disclosed by earlier exercises.

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Experimental

Preparation of Fish Oil Samples

Approximately 25 herring (*Clupea harengus harengus*) of commercial fishery size (25-30 cm fork length) were filleted, and fillets were minced in a Hobart Silent Cutter. The approximately 5 kg mince was mixed with 3 volumes (~15 L) of glass-distilled acetone and allowed to stand overnight. The extract was filtered through prewashed Whatman No. 1 paper, and the residue was re-extracted twice with 5 L glass-distilled acetone-hexane (1 + 1). The filtrates were pooled and mixed with glass-distilled water until the phases separated. The aqueous phase was washed twice with 500 mL portions of hexane which, after separation, were added to the original hexane layer. The solvent was removed under vacuum in a Buchler flash evaporator (~30°C), using dry ice-ethanol to cool the collecting reservoir. The resulting oil was stored under nitrogen at all times to minimize oxidative degradation. One-half of the oil was spiked with Aroclor 1254 (Monsanto Co., St. Louis, MO 63166) dissolved in a minimum volume of hexane to add 1.00 mg PCB/kg oil. After thorough mixing with a magnetic stirrer, 5-10 mL portions of each oil (spiked and unspiked) were flame-sealed in solvent-washed borosilicate glass ampules flushed with nitrogen. Extreme caution was used to prevent any of the oil from adhering to the heat-sealed area. These ampules were analyzed periodically by the coordinating laboratory to determine the stability of PCB content. Intercalibration kits, consisting of one ampule of each of the oils plus a vial of Aroclor 1254 from the same batch used to spike the oil, were prepared and distributed to the participating labo-

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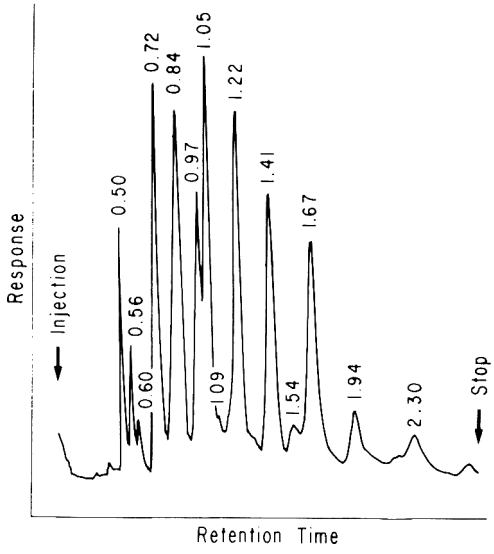

Figure 1. Chromatogram of supplied PCB standard (Aroclor 1254) in hexane. Conditions: isothermal at 200°C, argon-methane (95 + 5) carrier gas at 50 mL/min. Peaks used for PCB quantitation at retention times 1.22, 1.41, and 1.67.

ratories by air mail. Instructions regarding analysis and quantitation were issued to participants as indicated in the following 2 sections.

Separation of PCBs from Oil

The method of cleanup of the oils was entirely at the discretion of the participant and was, for the most part, the method normally used by the participant for routine analysis of PCBs in marine oils. The methods included column chromatography using Florisil, alumina, and/or silica gel, saponification, concentrated H_2SO_4, partition, and thin layer chromatography.

Quantitation of PCBs

For quantitation, packed column gas chromatography with electron capture detection (ECGC) was generally used. Several laboratories used glass capillary gas chromatography. Participants were requested to quantitate PCBs by using (a) their own standard, and (b) the supplied 1254 standard. Results based on 2 calculation methods were also requested: (a) the participant's usual method; and (b) another (the ICES method) using the sum of 3 prominent peaks of Aroclor 1254 as follows: composed of tetra- to hexachlorobiphenyls and eluting from a 180 cm × 4 mm glass column packed with 3% SP-2100 on 80–100 mesh Supelcoport at retention times relative to *p,p'*-DDE (R_{DDE}) of 1.22, 1.41, and 1.67 (see Figure 1).

Participants were also requested to prepare duplicate standard solutions of the supplied Aroclor 1254 on 2 different days and to report these results.

Calculation of Theoretical Plate Values

Theoretical plate counts were calculated by the coordinating laboratory from representative chromatograms of the spiked oil submitted by the participants. For this calculation, the peak with R_{DDE} = 1.67 (Figure 1) was used.

Statistical Treatment of Results

The results in the tables indicating the PCB ranges in unspiked and spiked oils as reported by the individual participants are presented on an as received basis. In subsequent tables, rounding was used where required to render data more amenable to statistical analysis. Because of the varying degrees of analytical investigation and insufficient information regarding appropriate weighting factors, it was difficult to analyze these data statistically. In those cases where statistical analysis of the data was performed, arithmetic means of applicable results were calculated.

Calculations of means, medians, standard deviations, and *t*-tests were performed on a Hewlett-Packard Model 97 programmable calculator. The statistical test for presence of systematic errors was done as recommended by Youden.

Results and Discussion

The great diversity of methods can readily be seen in Table 1. Tables 2 and 3 present all results received from the analysts, along with the coordinating laboratory's calculated recovery of 1.00 mg PCB/kg oil spike (Table 3). Reported levels of PCB in the unspiked oil ranged from 0.48 to 3.416 mg PCB/kg oil, while the spiked oil concentration ranged from 0.70 to 3.891 mg PCB/kg oil. Recovery of the 1.00 mg/kg spike, calculated from the data submitted by the authors, ranged from 0.22 to 1.364 mg/kg.

It should be noted that the figures in Tables 2 and 3 represent various levels of analytical investigation by the various laboratories. Not all analysts reported all 4 values based on the 2 PCB standards (the supplied one and the laboratory's own) and the 2 methods of calculation (the ICES method and the analyst's own). One participant (No. 1) used 3 chromatographic columns in the quantitation step and reported values for each column. In general, the participants did not indicate which value was the most likely value for the PCB level; therefore, Tables 2 and 3 con-

Table 1. List of methods used to determine PCBs

Lab.	Analytical procedure	Chromatograph method
1	Florisil cleanup, KOH removal of DDT group, peak height, 3 column confirmation	packed column
2	alumina cleanup, silica gel split, peak height	packed column
3	alumina cleanup, Florisil split, peak height	packed column
4	alumina cleanup, silica gel split, peak height, chromic acid confirmation	packed column
5	silica gel cleanup/split, peak height	packed column
6	Florisil cleanup, silica gel split, peak area	packed column
7	saponification, alumina cleanup, peak height, peak area	packed column, capillary GLC for individual isomers
8	Florisil cleanup, silica gel split, peak height	packed column
9	hexane/DMF/hexane, alumina, KOH, peak height	packed column
10	column extraction, Florisil cleanup/split, H_2SO_4 treatment, peak height, peak area	packed column capillary GLC
11	H_2SO_4 cleanup, perchlorination, peak area	packed column
12	H_2SO_4 cleanup, hexane extraction, peak height	capillary GLC
13	Florisil cleanup/split, chemical cleanup (KOH, CrO_3), peak height	packed column
14	alumina cleanup, silica gel split, peak height, peak area	packed column
15	alumina cleanup, silica gel split, peak height	packed column
16	alumina cleanup, silica gel split, peak height	packed column
17	Florisil cleanup/split, peak area	capillary GLC
18	alumina cleanup, silica gel split, peak area	capillary GLC packed column
19	saponification and perchlorination or alumina cleanup, silica gel split, peak height	packed column
20	alumina cleanup/split, peak area (?)	packed column
21	alumina cleanup/Florisil split, peak height	packed column
22	H_2SO_4 cleanup, peak height	packed column
23	TLC cleanup, peak height	packed column

tain the range of values reported. It must be emphasized that a narrow range does not necessarily reflect better performance but may simply reflect the degree of analytical investigation.

Statistical Interpretation of the Data

Table 4 lists the results for the unspiked oil separated into 4 groups according to the standard and method of calculation used. Only 8 participants reported results which could be divided into each of the 4 groups; these 8 are boldface. Arithmetic means ($\overline{X}_{4M}$) for each group were not significantly different (1.11–1.22 mg/kg) and coefficients of variation (CV) were all similar (32–41%). This indicates that use of either a common PCB standard or a common calculation method does not result in a significant decrease in interlaboratory variance. However, the grand mean ($\overline{X}_T$) based on the mean value for each of all the participants was 1.14 ± 0.56 mg/kg. $\overline{X}_T$ is not significantly different from $\overline{X}_{4M}$ but the CV is larger (49%). This indicates that laboratories which submitted less than the requested amount of data are, as a group, less able to obtain intercomparable results. This large CV is similar to that reported by Holden (4) for the fourth ICES organochlorine intercalibration in spite of the somewhat lower levels of PCB in the oil used in Holden's study.

The CV associated with $\overline{X}_{4M}$ (32–41%) compares favorably with that reported by Sawyer (5) for an environmentally incurred PCB residue in fish (4.5 mg/kg ± 20%) considering that (a) the PCB concentration in Sawyer's study was approximately 4 times higher than in our study, and (b) standardized methodology was employed in Sawyer's study. In comparison, Pavlou and Hom (1) reported a CV of ±25% for PCB in marine sediments, with most of the 10 participants in that study using similar methodology.

Results for the spiked oil (Table 5) essentially mimicked those for the unspiked oil, differing only in the lower coefficients of variation. This can be expected, considering the higher concentration of PCBs in the spiked oil and the similar standard deviations. As with the unspiked oil, there was no evidence to support the use of a common PCB standard or a common calculation method among the 8 laboratories submitting adequate data, since there were no significant differences among the 4 means (1.92–2.05 mg PCB/kg oil) or their CV values

Table 2. PCB levels reported for unspiked oil (mg/kg oil)

	ICES Aroclor 1254 std		Laboratory PCB std	
Lab.	ICES calcn	Lab. calcn	ICES calcn	Lab. calcn
1-1[a]	0.86	1.28	0.97	1.38
1-2[b]	0.83	0.84	0.90	0.93
1-3[c]	1.01	1.03	1.15	1.20
2-1[d]	1.050	1.000	1.015	0.970
2-2[e]	0.905	0.870	0.910	0.860
3	1.164	—	—	—
4	1.36, 1.58	1.46, 1.69	1.08, 1.26	1.17, 1.40
5	0.947	0.727	0.843	0.709
6-1[d]	—	0.934, 1.016	—	1.037
6-2[e]	—	1.030	—	—
7	1.000	—	0.950	—
7A[f]	—	—	—	0654[g]
8-1[d]	2.02	1.66	1.82	1.52
8-2[e]	2.14	1.72	1.93	1.61
9	—	0.606	—	—
10	0.763, 0.819	0.808, 0.866	0.817	0.865
10A[f]	1.014, 0.935	—	1.010	—
11	—	3.416[h]	—	3.210[h]
12-1[d]	—	1.29, 1.35, 1.44[i]	—	1.65
12-2[e]	—	1.28, 1.35	—	1.61
13	1.30, 1.71	—	—	—
	1.20, 1.44	—	—	—
	1.32, 1.39	—	—	—
	1.38, 1.38	—	—	—
14	0.732[j]	0.658[j]	0.667[j]	0.692[j]
	0.840[k]	0.790[k]	0.750[k]	0.800[k]
15	—	1.05	—	—
16	—	0.830, 0.841[l]	—	—
17	—	1.35, 1.36, 1.17	—	—
18	1.19, 1.19	1.305[m]	—	—
	0.82	—	—	—
18A[f]	—	—	—	0.725[g]
19	0.72	0.70[h]	—	0.74
20	—	1.084, 0.860	—	0.950, 0.860
21	1.079, 1.036	1.135, 1.119, 1.169[n]		—
	1.082	1.290, 1.243, 1.282[o]		—
		0.812, 0.746, 0.794[p]		—
22	—	0.48	—	—
23	1.80[q]	1.80[q] 1.60[q]	1.60[q]	

[a,b,c] Three different chromatographic columns.
[d,e] Two ICES intercalibration kits.
[f] Using capillary chromatography.
[g] Individual isomers.
[h] Based on perchlorination.
[i] Analyzed 1 month later.
[j] Peak height calculation.
[k] Peak area calculation.
[l] Repeated next day.
[m] Based on Aroclor 1260.
[n,o,p] Based on individual (order of elution) peaks as recommended by ICES.
[q] Repeated on 6 different days.

(19–24%). The grand mean $\overline{X}_T$ for the spiked oil was not significantly different from these 4 means but its CV (28%) was, like that for the unspiked oil, somewhat larger than the 4 individual means.

Calculation of the means after deletion of results differing from their grand means by more than 3 standard deviations (one laboratory in each set) did not significantly lower the means ($\overline{X}_{excl}$ = 1.07 ± 0.33 mg/kg for unspiked oil and 1.93 ± 0.41 mg/kg for spiked oil) but did lower the CV values (31% and 21%, respectively) to levels associated with the 8 laboratories reporting adequate data.

Median values for the unspiked and spiked oils were determined (1.02 and 1.94 mg/kg, respectively). The medians are not significantly different from $\overline{X}_{excl}$ for each oil. Similarly, the

Table 3. PCB levels reported for spiked oil (mg/kg oil)

	ICES Aroclor 1254 std		Laboratory PCB std		
Lab.	ICES calcn	Lab. calcn	ICES calcn	Lab. calcn	Diff.[r]
1-1[a]	1.79	2.14	2.00	2.30	
1-2[b]	1.54	1.67	1.69	1.85	
1-3[c]	1.92	1.88	2.18	2.17	0.71–1.03
2-1[d]	1.590	1.540	1.560	1.515	
2-2[e]	1.570	1.470	1.515	1.440	0.540–0.665
3	1.696	—	—	—	0.532
4	2.39, 2.27	2.66, 2.43	1.91, 1.81	2.19, 2.00	0.69–0.97
5	1.610	1.466	1.507	1.441	0.663–0.739
6-1[d]	—	1.899, 2.032	—	1.980	
6-2[e]	—	1.952, 1.931	—	1.920	0.922–1.016
7	1.940	—	1.910	—	0.860–0.940
7A[f]	—	—	—	1.145[g]	0.591[s]
8-1[d]	2.64	2.23	2.37	2.04	
8-2[e]	3.06	2.54	2.76	2.38	0.52–0.92
9	—	1.827	—	—	1.221
10	1.627, 1.747	1.683, 1.806	1.744	1.803	0.864–0.938
10A[f]	2.012, 1.854	—	2.010	—	0.919–0.998
11	—	3.891	—	3.785	0.475–0.575[t]
12-1[d]	—	2.17, 2.27, 2.42[i]	—	2.75	
12-2[e]	—	2.33, 2.22	—	2.86	0.88–1.25
13	2.37, 2.42, 2.49, 2.55	—	—	—	—
		—	—	—	1.07[u]
14	2.000[j]	1.956[j]	1.821[j]	2.056[j]	
	2.014[k]	1.956[k]	1.800[k]	1.980[k]	1.050–1.364
	—	2.15	—	—	1.10
16	—	1.888, 1.913[l]	—	1.956	1.058–1.095
17	—	1.790, 2.130	—		0.667[d]
18	1.95, 1.97, 1.40	2.07[m]	—	—	0.670–0.780
		—	—	—	(0.765)[m]
18A[f]	—	—	—	1.295	0.570[s]
19	1.48	1.53[n]	—	1.67	0.76–0.89
20	—	2.129, 2.005	—	1.867, 1.757	0.960–1.095
21	1.875, 1.875	1.844, 1.864, 1.754[n]	—		—
	1.910	2.093, 2.098, 2.49[o]	—		0.677–0.870
		1.687, 1.664, 1.635[p]			
22	—	0.70	—	—	0.22
23	2.60[q]	2.70[q]	2.40[q]	2.40[q]	0.60–1.00

[a–q] As in Table 2.

[r] The oil was spiked with 1.00 μg PCB (Aroclor 1254)/g. These sets of values were calculated from paired sets of data submitted by individual laboratories. There is no direct relationship between maxima and minima of spiked and unspiked oils and maximum and minimum differences.

[s] Some isomers.

[t] Based on decachlorobiphenyl.

[u] Difference of the means.

overall recovery of the spike (difference of 2 grand means for laboratories) was 0.87 ± 0.23 and the median value was 0.91. This suggests we are dealing with a population of results distributed in a normal manner.

One further point should be noted. No differences were found in the standard deviation of means of unspiked and spiked oil values determined by any of the above calculations or groups of laboratories. This suggests that the majority of the variance arises from the methodology at some point in which the spiked and unspiked oil would suffer the same apparent losses or gains of PCBs within each laboratory. The standard deviation of $\overline{X}_{excl}$ is composed of the standard deviation of laboratory precision and the standard deviation of the systematic errors and these are equivalent for both oils. According to Youden (6), this means that neither oil induces a systematic or laboratory precision change. It is possible to interpret this study by using each laboratory's mean value and eliminating laboratories using perchlorination or capillary (PCB isomers) techniques, because now, to a first approximation, we have a "common" analytical method (gas chromatography using a mixed PCB standard) and evidence that the systematic error is the same for each oil. From the values from

Table 4. Mean levels of PCB reported in unspiked oil (mg/kg oil)

Lab	ICES Aroclor 1254 std		Laboratory PCB std		Lab. mean
	ICES calcn	Lab. calcn	ICES calcn	Lab. calcn	
1	0.90	1.05	1.01	1.17	1.03
2	0.98	0.94	0.96	0.92	0.95
3	1.16	—	—	—	1.16
4	1.47	1.58	1.17	1.29	1.38
5	0.95	0.73	0.84	0.71	0.81
6	—	0.99	—	1.04	1.02
7	1.00	—	0.95	—	0.98
8	2.08	1.69	1.88	1.57	1.81
9	—	0.61	—	—	0.61
10	0.79	0.84	0.82	0.87	0.83
10A	0.98	—	1.01	—	1.00
11	—	3.42	—	3.21	3.31
12	—	1.34	—	1.63	1.51
13	1.39	—	—	—	1.39
14	0.79	0.72	0.71	0.75	0.74
15	—	1.05	—	—	1.05
16	—	0.84	—	0.86	0.85
17	—	1.29	—	—	1.29
18	1.07	—	—	1.31[a]	1.19
19	0.72	0.70	—	0.74	0.72
20	—	0.97	—	0.91	0.94
21	1.07	1.14, 1.27[b] 0.78	—	—	1.07
22	—	0.48	—	—	0.48
23	1.80	1.80	1.60	1.60	1.70
	$\bar{X}_{4m}$[c] = 1.22 ± 0.50[8][d] (41%)[f]	1.17 ± 0.45 (38%)	1.12 ± 0.41 (37%)	1.11 ± 0.35 (32%)	$\bar{X}_T$[e] = 1.14 ± 0.56[24] (49%) $\bar{X}_{excl}$[g] = 1.07 ± 0.33[23] (31%) Median = 1.02

[a] Based on Aroclor 1260.
[b] Based on individual peaks of Aroclor 1254.
[c] Mean ± SD (standard deviation) of boldface laboratories.
[d] Number of laboratories included in calculation.
[e] Grand mean of all laboratory means.
[f] Coefficient of variation (CV).
[g] Mean excluding all results > $\bar{x}_T$ ± 3 SD (i.e., $x > 2.82$: Lab. 11).

Tables 4 and 5, which had been used to calculate $\bar{X}_{excl}$ for each oil, we have determined that the precision (intralaboratory) standard deviation (S_r) is 0.654 while the overall standard deviation (S_d) is 2.2461. The F-ratio is 11.79, which shows that systematic error is the predominant source of the variance, rather than intralaboratory precision ($P \ll 0.01$) (6).

Sources of Error

It should be noted that some laboratorys' duplicate standard solutions show as much as 6% difference, which could contribute to the systematic error and indicates that some participants had instrumentation problems (balance, syringe, gas chromatograph, glassware).

It is possible that the chromatographic stationary phase may have some major effect on the quantitation of PCBs in the oils. This is demonstrated by the results submitted by Laboratory 1 which analyzed the PCB fraction by using the 3 different silicone stationary phases, SP-2401, OV-101, and OV-105, and reported results which were quite dependent on the stationary phase used. When the means reported by laboratories (Nos. 1B, 3, 5, 7, 10, 13, 18, 21) using high performance polydimethylsiloxane stationary phases (OV-1, OV-101, and SP-2100) are compared with the means ($\bar{X}_{excl}$) from Tables 4 and 5, no significant differences are noted in the 2 means for either the unspiked or the spiked oil. However, it should be noted that the coefficients of variation of the results from laboratories which employed a high grade of silicone stationary phase were substantially lower than that of the grand mean. This suggests that analysts should consider use of such stationary phases.

It is also possible that laboratories employing highly efficient packed chromatographic columns would, as a group, perform better than the

Table 5. Mean levels of PCB reported in spiked oil (mg/kg oil)

Lab.	ICES Aroclor 1254 std		Laboratory PCB std		Lab. mean
	ICES calcn	Lab. calcn	ICES calcn	Lab. calcn	
1	1.75	1.90	1.96	2.11	1.93
2	1.58	1.51	1.54	1.48	1.53
3	1.70	—	—	—	1.70
4	2.33	2.54	1.86	2.10	2.21
5	1.61	1.47	1.51	1.44	1.51
6	—	1.96	—	1.92	1.94
7	1.94	—	1.91	—	1.93
8	2.85	2.39	2.57	2.39	2.55
9	—	1.83	—	—	1.83
10	1.69	1.75	1.74	1.80	1.74
10A	1.93	—	2.01	—	1.97
11	—	3.89	—	2.79	3.84
12	—	2.24	—	2.81	2.53
13	2.46	—	—	—	2.46
14	2.01	1.96	1.81	2.02	1.95
15	—	2.15	—	—	2.15
16	—	1.91	—	1.96	1.93
17	—	1.96	—	—	1.96
18	1.77	—	—	2.07[a]	1.92
19	1.48	1.53	—	1.63	1.55
20	—	2.07	—	1.82	1.94
21	1.89	1.82, 2.08[b] 1.662	—	—	1.87
22	—	0.70	—	—	0.70
23	2.60	2.70	2.40	2.40	2.53
	$\bar{X}_{4m}$[c] = 2.05 ± 0.49[8][d] (24%)[f]	2.03 ± 0.47 (23%)	1.92 ± 0.38 (20%)	1.97 ± 0.37 (19%)	$\bar{X}_T$[e] = 2.01 ± 0.56[24] (28%) $\bar{X}_{excl}$[g] = 1.93 ± 0.41[23] (21%) Median = 1.94

[a,b,c,d,e,f] As in Table 4.
[g] Mean excluding all results > $\bar{X}_T$ ± 3 SD (i.e., x > 3.58: Lab. 11).

whole group or at least better than analysts employing less efficient packed columns. When results from laboratories using columns with efficiencies of greater than 2000 theoretical plates (Nos. 6, 13, 14, 19, 20, and 21) are compared with those using columns of less then 2000 plates (Nos. 1, 2, 3, 4, 5, 7, 8, 9, 10, 15, 16, 18, and 22) and to $\bar{X}_{excl}$, no significant differences are found.

We compared results on the use of Florisil or alumina as cleanup agent (Table 6). Results of a t-test suggest a trend toward higher mean values for both oils when cleaned up on Florisil. It is difficult to assess the importance of the difference in results between Florisil and alumina. The Florisil results are not significantly different from the 4 results obtained by capillary chromatography, not unexpectedly because 3 of 4 capillary users used Florisil, and the fourth, a sulfuric acid cleanup. The results may be interpreted as a selective loss of PCB on alumina but this is contradicted by the spike recovery (difference) figure for alumina which did not differ significantly from that of Florisil (91 and 88%, respectively). It is possible that Florisil allows some interfering material to co-elute with PCB which cannot be separated by capillary chromatography. However, this seems highly unlikely considering the efficiency of the capillary columns used. What is more probable is more effective retention of lipid components by Florisil. Visual inspection of the submitted chromatograms indicated a high degree of correlation between the occurrence of negative peaks (below baseline) and the use of alumina for cleanup. These peaks are caused by easily ionized (electron-donating) biogenic hydrocarbons such as pristane, the presence of which was reported by one participant (No. 20) using a flame ionization detector. Ackman (7) also has reported the presence of large amounts of pristane and octadecane in herring as well as smaller quantities of other nonsaponifiable hydrocarbons. While negative peaks are most evident in the early part of the chromatograms where relatively few PCB isomers elute, it is entirely probable that the later-eluting PCB components

Table 6. Comparison of PCB concentrations (mg/kg) using alumina and Florisil for cleanup

Lab.	Unspiked oil	Spiked oil	Diff.
	Alumina		
2	0.95	1.53	0.58
3	1.16	1.70	0.54
4	1.38	2.21	0.84
7	0.98	1.93	0.96
9	0.61	1.83	1.22
14	0.74	1.95	1.21
15	1.05	2.15	1.10
16	0.85	1.93	1.08
18	1.19	1.92	0.73
19	0.72	1.55	0.83
20	0.94	1.94	1.00
21	1.07	1.87	0.80
$\bar{X}$ alumina = 0.97 ± 0.22 (23%)		1.88 ± 0.21 (11%)	0.91 ± 0.23 (25%)
$\bar{X}_{excl}$ = 1.07 ± 0.33 (31%)		1.93 ± 0.41 (21%)	
	Florisil		
1	1.03	1.93	0.90
6	1.02	1.94	0.92
8	1.81	2.55	0.74
10	0.83	1.74	0.91
10A	0.99	1.97	0.98
13	1.39	2.46	1.06
17	1.29	1.96	0.67
$\bar{X}$ Florisil = 1.19 ± 0.33[a] (28%)		2.08 ± 0.30[a] (15%)	0.88 ± 0.13 (15%)
$\bar{X}_{excl}$ = 1.04 ± 0.31 (30%)		1.90 ± 0.39 (21%)	

[a] $0.1 < P < 0.05$ indicates that this mean is significantly different from the corresponding alumina mean.

most often used for quantitation are superimposed on such negative peaks, the overall result of which would be a positive peak of diminished size.

Additional information can be gained by inspection of Figure 2. This Youden plot of each laboratory's result for the unspiked oil vs that of the spiked oil visually identifies laboratories which submitted consistently high or consistently low results compared with the median

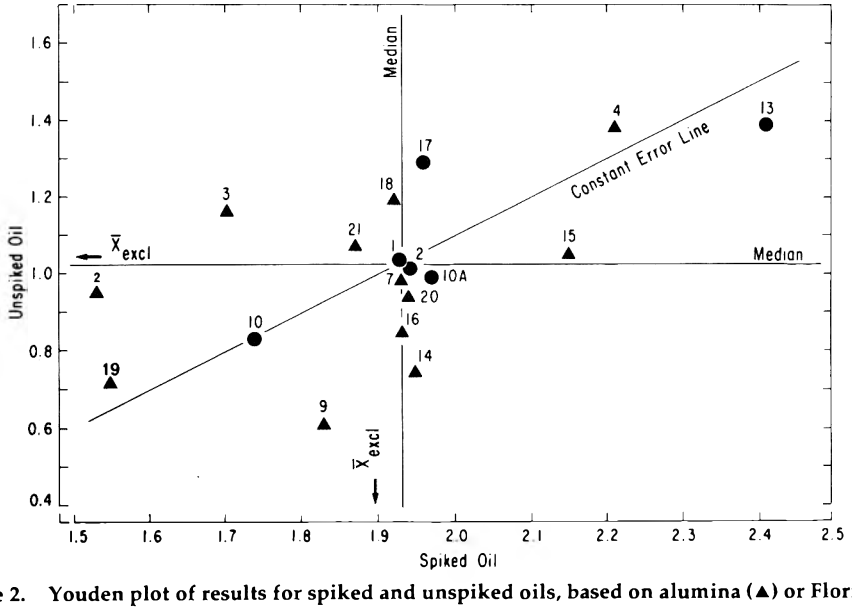

Figure 2. Youden plot of results for spiked and unspiked oils, based on alumina (▲) or Florisil (●).

levels of all laboratories for each oil (6). Laboratory results which fall in the upper left or lower right quadrant are inconsistent. It is interesting that of 6 laboratories falling within the inconsistent areas, 5 used alumina as cleanup adsorbent.

A line (constant error line) with a slope of 1 passing through the median intercept may be used to judge the degree by which a laboratory's results differ from those in which both results differ from the intercept of the 2 median lines by the same amount. A laboratory result falling along the constant error line in the upper right quadrant has reported results which have higher values by a constant amount than the median values while those in the lower left quadrant report values a constant amount lower than the median values. Visual inspection of the figure suggests that laboratories using Florisil as a cleanup agent fall closer to this constant error line than do those laboratories using alumina for cleanup. It should also be noted that the use of a correction factor for each laboratory upon which to compare data from different laboratories can only be justified for those laboratories falling close to the constant error line, because use of such a correction factor would only correct for a constant error.

Conclusions and Recommendations

The results of this collaborative study indicate that problems still exist in the determination of PCBs in fish oils and in interlaboratory comparisons of results, especially when a variety of methods is used.

While we have identified that systematic error is the most likely source of the observed variance, we are unable to state whether such systematic error is due to differences inherent in the procedures used by the various participants or by differences that would be inherent in each laboratory's application of a common method. There is evidence to support the former possibility and the answer to this question would come from an intercalibration study using, as far as possible, identical methods. Results of Sawyer's collaborative study (5) indicate that a somewhat lower CV can be obtained for fortified samples through use of a standard method; however, in the case of an environmentally incurred PCB residue in fish, the value obtained was 4.5 mg/kg ± 20%. Therefore, it would seem that the results of our study compare fairly well to the state-of-the-art, considering that we used a great variety of analytical methods, and that we were determining PCB concentrations one-quarter to one-half as great as that mentioned above.

Our study corroborates that of Sawyer, who reported that use of a common calculation method does not result in an increase in precision when commercial PCB mixtures are used as standards.

An intralaboratory study comparing alumina, Florisil, and concentrated sulfuric acid is warranted. The role of hydrocarbon interferents, both physiologically based and environmentally incurred, in affecting the determination of PCB and other organochlorine contaminants in herring and other marine and freshwater fish, should be elucidated. The results of our study suggest that estimates of PCB in herring, obtained by participants using the alumina procedure, may be negatively in error by as much as 20%.

Negative peak interference could account for the differences in results reported by Laboratory 1 (different results from different stationary phases). The phases used (SP-2401, OV-101, and OV-105) vary in degree of polarity, with OV-105 exhibiting the largest McReynold's constants. The retention times of the interfering biogenic hydrocarbons would vary considerably with respect to PCB components on these phases, giving rise to the varied PCB values.

If the use of packed columns is to be continued, analysts should use as highly efficient columns as possible. The use of high performance stationary phases may improve the results somewhat.

Finally, we suggest that improvement would occur should PCBs be determined on the basis of individual isomers. Sawyer (3) found that the precision of determination of PCBs in milk and chicken fat was improved by quantitation based on individual peaks and using a synthetic standard rather than the commercial PCB mixtures. In this study, packed columns were used. The use of capillary columns should improve interlaboratory precision still more, yielding results less in error from interfering peaks than packed columns. In the current study, 6 participants used capillary chromatography, 2 of which reported concentrations of PCB components in the oils. The other 4 participants reported mean values for the spiked and unspiked oils which were higher than the mean values $\overline{X}_{excl}$ reported for these oils in Tables 4 and 5. Also, the CV of the spiked results was reduced by half. The significance of this observation is difficult to judge because only a small number of capillary chromatographs was involved. Laboratory 10

analyzed the oils by both packed and capillary column gas chromatography and reported the same observation, i.e., higher results from the latter technique. This is inconsistent with findings generated by the author who found that the PCB levels in hexane extracts (stored for 9 months in a refrigerator at 3.5°C) of the oils were lower by capillary chromatography than by packed column. This effect was greater for the unspiked oil than for the spiked. While the capillary column would magnify differences in concentrations of chlorinated biphenyls between unspiked and spiked oils, further work is required to resolve these inconsistencies. Another intercalibration exercise using capillary gas chromatography has been carried out (8) and will be the subject of a subsequent paper.

Acknowledgments

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Gas-Liquid Chromatographic Determination of Polychlorinated Biphenyls and a Selected Number of Chlorinated Hydrocarbons in Serum

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A method is proposed for concurrently determining the levels of multiple intact exogenous compounds in serum, particularly polychlorinated biphenyls (PCBs) as Aroclor (AR) 1254 and chlorinated hydrocarbons (CHs). Bovine serum pools containing in vivo-bound PCBs (as AR 1254) and in vitro-spiked CHs are used to evaluate the method, which encompasses serum denaturation with methanol, mixed solvent extraction, multiple solvent fractionation from activated silica gel, and determination by electron capture gas-liquid chromatography. Mean recoveries of the in vitro-spiked 9 CHs at levels of 2.0-29.1 ppb ranged from 52.8 to 98.4% from trial environmental pools; mean recoveries of the in vivo-bound PCBs (as AR 1254) were 114.1 and 92.6% at levels of 10 and 50 ppb, respectively.

Since the first analytical evidence was found that DDT is stored in the human body (1), increasing numbers of chemicals have been discovered in the food chain (2); many of these chemicals are stored in humans (3, 4). The levels of these compounds must be monitored periodically so that trends and levels of exposure can be assessed. Methods must be developed that permit a series of intact chemicals to be monitored routinely.

Because of the nature of the major environmentally dispensed substances (chlorinated hydrocarbons (CHs), chlorinated biphenyls) known to be stored in humans, it is difficult to resolve these compounds so they may be ultimately measured. Analytical approaches have been described for the concurrent analyses of polychlorinated biphenyls (PCBs) and CHs in sources to which humans are exposed, e.g., food (5), water sediment and bottom material (6), and air (7). However, few accounts have been published regarding attempts to concurrently analyze multiple intact compounds in blood serum. In our review of the scientific literature, we found no discussion of many of the problems associated with this analysis. We did find descriptions of analyses for either CHs (8-10) or PCBs (11-14) in serum. With few exceptions, the determinations of PCBs in serum have been predicated on a pretreatment of the serum to preclude or minimize the contribution of the CHs. Studies in which intact organochlorine compounds and PCBs have been reported in blood have been limited to (a) evaluation of these compounds in toxemia of pregnancy, by Wassermann et al. (15); (b) assessment of these compounds in mother and fetus during labor (16) and after normal delivery, by Polishuk et al. (17); (c) determination of these compounds in cachectic patients and healthy persons, by Hesselberg and Scherr (18); and (d) evaluation of serum levels of PCBs (19) and DDTs (20) in residents of Triana, Alabama, by Kreiss et al.

Because the first 4 publications mentioned above (15-18) are not method papers, the quality of the separation which preceded the simultaneous determination of PCBs and CHs is not described. In the approach used by Kreiss et al. (19), silica gel was impregnated with silver nitrate to separate the PCBs (as Aroclor 1260) from DDT and metabolites; however, the DDT and DDD isomers could not be recovered intact because they were dehydrohalogenated. Instead, DDT and metabolites were determined after column chromatography, with silica gel used as the adsorbent (20); therefore, the DDT concentration values, specifically the isomers of DDD and DDT, contained contributions from PCBs. Needham et al. (21) reported using these dual adsorption columns so that the CHs and PCBs could be quantitated by difference.

The method presented here involves serum denaturation with methanol, mixed solvent extraction, multiple solvent fractionation from activated silica gel, and subsequent determination by electron capture gas-liquid chromatography (22, 23). The problems inherent in applying this methodology to bovine serum

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pools containing in vitro-spiked CHs and in vivo-bound PCBs as AR 1254 will be discussed.

Preparation of Pool Material

To evaluate the procedure, we prepared serum pools containing in vivo-bound PCBs and a selected number of in vitro-spiked CHs. The pool material was prepared to reflect the general population in terms of types and concentrations of CHs and PCBs present. The levels of CHs selected were mean or maximum levels obtained from the Environmental Protection Agency (EPA) (Richard Kutz, December 1978), and PCB levels were indicative of the mean level and 5 times the mean level obtained in other studies involving populations not known to be abnormally exposed to PCBs (PCBs/PBBs serum screening of Michigan residents by the Michigan Department of Public Health and the Centers for Disease Control (CDC)).

The source of the in vivo-bound PCBs (as AR 1254) was a cow that had been given oral doses of AR 1254 for 14–21 days, allowed to approach equilibrium of storage in the absence of dosing, and then bled. After we separated the serum from the whole blood, we determined its PCB concentration. The following CH standards (purity 98–100%) were obtained from the Pesticide Repository of the EPA, Research Triangle Park, NC: hexachlorobenzene, hexachlorocyclohexane, oxychlordane, heptachlor epoxide, *trans*-nonachlor, *p,p'*-DDE, dieldrin, *o,p'*-DDT, and *p,p'*-DDT. Appropriate amounts of these individual standards in isooctane were combined, the solvent was evaporated, and the residue was redissolved in acetone to comprise the spiking solution.

The trial environmental pools (TEPs) were prepared as follows:

TEP 3: To a pool of essentially CH- and PCB-free bovine serum, an amount of serum from the cow dosed with AR 1254 was added to yield a fortification level of approximately 50 ppb (ng/mL) PCBs.

TEP 2: An aliquot of TEP 3 (after 72 h of mixing at 0°C) was removed and added to a pool of essentially CH- and PCB-free bovine serum to yield a fortification level of approximately 10 ppb (ng/mL) PCB. This pool was allowed to mix for 72 h at 0°C. The 9-component CH spiking solution (25 mL) was added to each of the above pools (1% of the final volume) and allowed to mix for an additional 72 h at 0°C.

TEP 1: To a pool of essentially CH- and PCB-free bovine serum, acetone (25 mL) was added (1% of the final volume) and allowed to mix for 72 h at 0°C.

We saw no evidence of a protein precipitate before any of the 3 pools were sterilized. All pools were sterilized by using a Millipore Filtration System, dispensed into 3-dram vials in a sterile environment, and stored at −30°C.

METHOD

Apparatus

(a) *Analytical system, PCBs.*—A Varian 3700 gas-liquid chromatograph (GLC) (Walnut Creek, CA) equipped with a Hewlett-Packard Model 7671A automatic injector (Avondale, PA), a constant current ^{63}Ni electron capture detector (ECD), and a Perkin-Elmer Model 56 strip-chart recorder were used. The chromatograph was controlled and the data were calculated by a Varian CDS 111(c) microprocessor. Operating conditions: temperatures (°C)—column 195, detector 330, injection port 250; nitrogen flow 20 mL/min; ECD range 10, and recorder attenuation ×8. Gas chromatographic column, 6 ft × 2 mm (id) glass, packed with performance-tested 3% SE-30 on 80–100 mesh Gas-Chrom Q.

(b) *Analytical system, CHs.*—A Varian 2100 GLC instrument equipped with a $^{3}H(Sc)$ electron capture detector and a Varian Model A-25 strip-chart recorder were used. Operating conditions: temperatures (°C)—column 200, detector 285, injection port 225; nitrogen flow 37 mL/min; electrometer range 10^{-10}; and attenuation ×16. Gas chromatographic column, 6 ft × 4 mm (id) glass, packed with 1.5%/1.95% OV-17/QF-1 on 80–100 mesh Chromosorb WHP.

(c) *Chromatographic column.*—Glass, 28 cm × 9 mm, equipped with 50 mL reservoir and polytetrafluoroethylene (PTFE) stopcock (Tudor Scientific Glass, Belvedere, SC).

Reagents

(a) *Solvents.*—Pentane, *n*-hexane, methanol, benzene, and ethyl ether were all distilled-in-glass grade (Burdick & Jackson, Muskegon, MI).

(b) *Sodium sulfate.*—Anhydrous, reagent grade. Wash with hexane and oven-dry continuously at 130°C. Remove from oven and let cool under desiccation before use.

(c) *Silica gel.*—Weigh 20 g silica gel (Woelm 70–150 mesh) into beaker, cover with aluminum foil (shiny side up; punch several holes in foil), and let stand in 200°C oven 8 h. Let cool overnight under desiccation. Transfer to Erlenmeyer flask containing 75–150 mL pentane. Hand-

rotate flask to rid mixture of any air bubbles, and seal with PTFE-lined screw cap.

Extraction

Pipet 4 mL serum into culture tube (16 × 125 mm with PTFE-lined cap), and add 2 mL methanol. Swirl briefly on vortex mixer. Extract serum with 5 mL hexane–ethyl ether (1 + 1) on rotary mixer (15 min at 50 rpm), centrifuge at 1800 rpm for 6 min, and then remove supernatant extract. Repeat extraction twice. Transfer each solvent extract to culture tube (20 × 150 mm with PTFE-lined cap). Concentrate combined extracts under gentle stream of nitrogen to low volume (0.5 mL).

Adsorption Chromatography

Prepare adsorption chromatography (9 mm id) column equipped with PTFE stopcock by adding, in order: silanized glass wool plug, 10 mm sodium sulfate, pentane to height of 125 mm, silica gel as a slurry from a shortened 5 mL (graduated in 1/10 mL) serological pipet to a height of 100 mm with settling, and 10 mm sodium sulfate.

Partially fill reservoir with pentane, and mark level. Adjust stopcock so that flow is 3 mL/min. During this adjustment, solvent also serves as prewash. Once flow is set, process continues without interruption. When level of pentane reaches top of sodium sulfate layer, transfer concentrated sample extract in 0.5 mL hexane to top of column. Rinse sample tube 3 times with 0.5 mL pentane per wash, and transfer wash to head of column. Once sample has been completely transferred, fill reservoir with pentane to previously marked level, and maintain this level during collection of fractions I, II, and III, each consisting of 15 mL eluate. Collect fractions in 15 mL conical centrifuge tubes that are calibrated to 1 mL and 15 mL. After fraction III is collected, let pentane eluant drain (referred to as pentane interface) into 20 × 150 mm culture tube. Then use benzene as eluant. Collect two 15 mL benzene fractions and designate them as IV and V. Reduce each of fractions I, II, and III and pentane interface to <1.0 mL in a 25°C water bath with gentle stream of nitrogen. Reduce fractions IV and V each to <1.0 mL in a 45°C water bath with a gentle stream of nitrogen. Reconstitute all fractions to 1.0 mL with hexane or isooctane.

Gas Chromatography

Polychlorinated biphenyls.—Generate necessary calibration factors by using Webb and McCall (24) Aroclor composition data for 3 μL injections of AR 1254 at 4 concentration levels ranging from 400 to 40 pg/μL. Once calibration factors are established, inject 3 μL of fractions II and III.

Chlorinated hydrocarbons.—Analyze all eluates, fractions I–V, and interface quantitatively by comparing responses of unknowns with appropriate concentration levels of the 9-component CH standard contained in hexane or isooctane. Analyze fractions II and III by using analytical system for CHs, because adsorption chromatography does not consistently preclude the elution of HCB, *trans*-nonachlor, and *p,p'*-DDE in one or more of these fractions.

Results and Discussion

Characterized values for TEPs (PCBs) were established through a series of 20 analytical runs in which fractions II and III were analyzed. In 5 of the 20 analytical runs, characterized values for the chlorinated hydrocarbons were also established through the analysis of fractions I–V and the interface. An analytical run consisted of the results of analyzing the extracts of duplicate samples taken from one vial each of TEPs 1, 2, and 3.

As shown in Table 1, most of the PCBs elute in

Table 1. Typical elution pattern from silica gel for CHs and PCBs found in TEPs 2 and 3 [a]

Fraction	HCB	β-HCCH	Oxychlordane	Heptachlor epoxide	*trans*-Nonachlor	*p,p'*-DDE	Dieldrin	*o,p'*-DDT	*p,p'*-DDT	PCBs as AR 1254
I	+	0	0	0	0	0	0	0	0	0
II	0	0	0	0	0	+ (11)	0	0	0	+ (82)
III	0	0	0	0	+ (17)	+ (64)	0	0	0	+ (18)
Interface	0	0	0	0	+ (63)	+ (22)	0	+ (66)	+ (18)	0
IV	0	+	+	+	+ (20)	+ (2)	+ (77)	+ (34)	+ (82)	0
V	0	0	0	0	0	0	+ (23)	0	0	0

[a] Average percent found in each fraction given in parentheses.

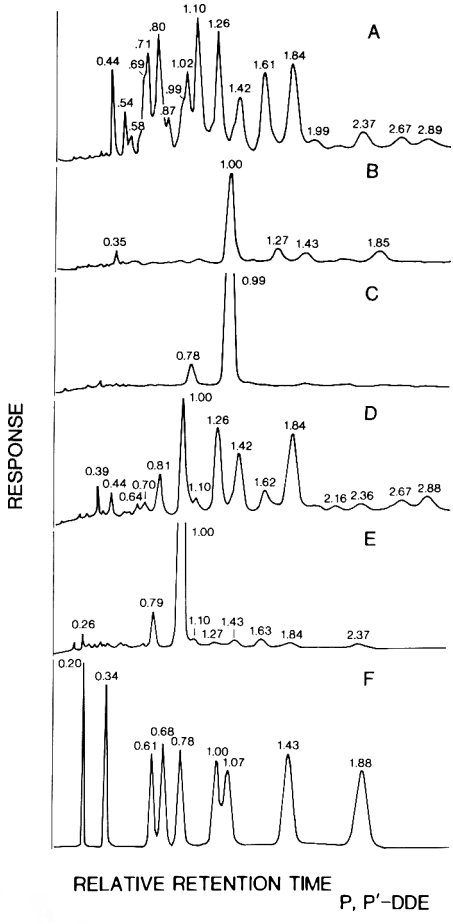

Figure 1. A, Aroclor 1254 standard (2100 pg injected); B, TEP 2 Fraction II; C, TEP 2 Fraction III; D, TEP 3 Fraction II; E, TEP 3 Fraction III (19 mg injected, all fractions); F, 9-component chlorinated hydrocarbon standard (pg injected, RRT_{DDE}): HCB (48.41 pg, 0.20); β-HCCH (199.28 pg, 0.34); oxychlordane (94 pg, 0.61); heptachlor epoxide (95.88 pg, 0.68); *trans*-nonachlor (113.74 pg, 0.78); *p,p'*-DDE (97.76 pg, 1.00); dieldrin (96.82 pg, 1.07); *o,p'*-DDT (287.64 pg, 1.43); *p,p'*-DDT (293.28 pg, 1.88). For GLC conditions, see text.

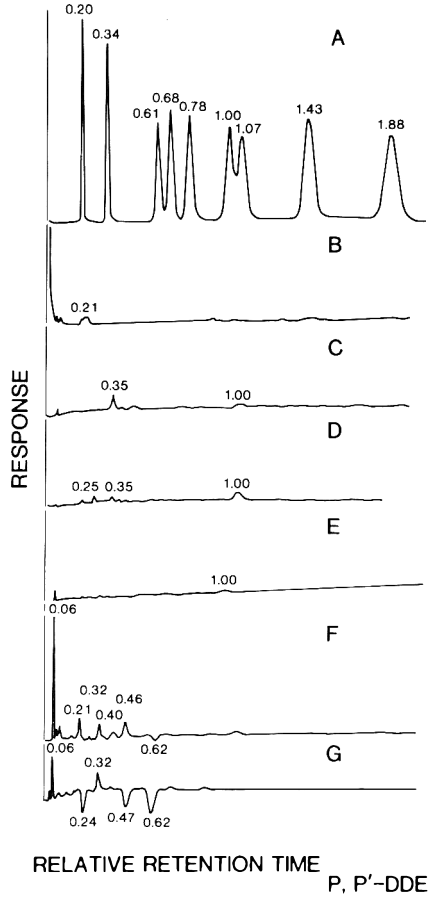

Figure 2. A, 9-component chlorinated hydrocarbon standard (concentrations and identities as in Figure 1); B, TEP 1 Fraction I; C, TEP 1 Fraction II; D, TEP 1 Fraction III; E, TEP 1 interface; F, TEP 1 Fraction IV; and G, TEP 1 Fraction V (19 mg injected, all fractions).

fraction II; a small percentage elutes in fraction III. Of the CHs that we evaluated, only *p,p'*-DDE eluted in fraction II, and only *p,p'*-DDE and *trans*-nonachlor eluted in fraction III. The only other CH that eluted either with or before PCBs was hexachlorobenzene; however, these 3 CHs can be discerned from PCBs by electron capture gas chromatography, either by the intensity of the peak (*p,p'*-DDE) or by retention times (HCB and *trans*-nonachlor). Gas chromatograms (under CH conditions) illustrative of total elution of PCBs (as AR 1254) and selected CHs are shown in Figures 1, 2, and 3. Gas chromatograms of fraction V contained the most extraneous peaks. Fortunately, the compound (dieldrin) that elutes in this fraction is well resolved from the contaminant(s). These traces again demonstrate that most CHs elute in fraction IV and most PCBs in fraction II. Although the mixed-phase column indicated the presence of several CHs in TEP 1 (Figure 2), only *p,p'*-DDE was confirmed by using a dual column technique. Column blank evaluations indicate that most of the peaks observed in fractions I–V of TEP 1 were attributable to contaminants from the silica gel.

Electron capture detection of contaminants resulting from elution of adsorbents of this type is not new (25, 26). We have applied to silica gel the solvent pretreatment techniques that had been used for silicic acid (27) and found that at

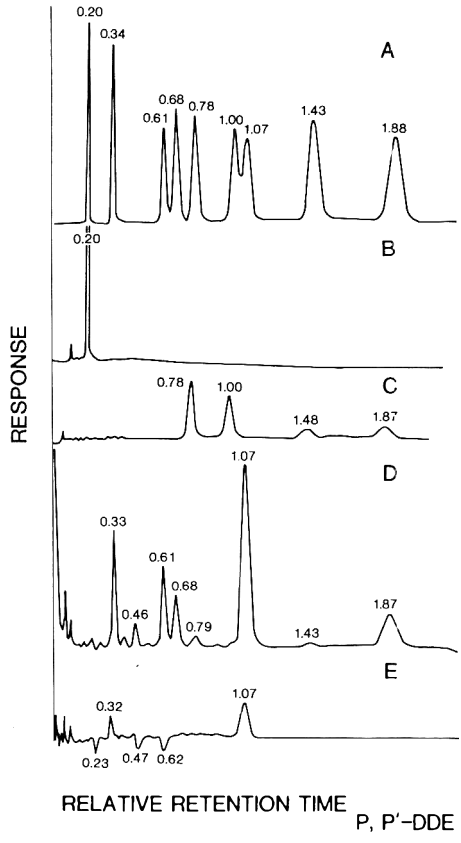

Figure 3. A, 9-component chlorinated hydrocarbon standard (concentrations and identities as in Figure 1); B, TEP 2 Fraction I; C, TEP 2 interface; D, TEP 2 Fraction IV; and E, TEP 2 Fraction V (19 mg injected, all fractions).

the levels at which we need to operate, even with pretreatment those solvent systems necessary to elute certain CHs from this type of absorbent invariably result in a measurable background. We have considered a cleanup procedure for this adsorbent somewhat analogous to one previously proposed (28) as a possible remedy for the undesirable contaminants observed in fraction V; however, its effect has not been evaluated.

Recovery of added pesticides (Table 2) ranged from 56.3% for β-HCCH to 98.4% for *o,p'*-DDT in TEP 2 and from 52.8% for β-HCCH to 96.4% for *o,p'*-DDT in TEP 3. Although in vitro spikes of the CHs are identical in these pools, the recovery is invariably higher in TEP 2. Our recoveries for some of the CHs were equal to, although most often not better than those reported in the literature in which parallel analytical techniques were used (10, 29, 30).

Table 2. Recovery (%) of in vitro-spiked chlorinated hydrocarbons[a]

CH	Spike, ppb	TEP 2	TEP 3
HCB	6.2	58.2 ± 5.0	56.0 ± 9.4
β-HCCH	8.4	56.3 ± 4.0	52.8 ± 3.6
Oxychlordane	4.0	78.6 ± 4.1	74.4 ± 6.6
Heptachlor epoxide	2.0	79.8 ± 5.8	76.2 ± 5.6
trans-Nonachlor	4.8	89.6 ± 4.8	85.0 ± 7.9
p,p'-DDE	29.1	63.0 ± 3.6	62.4 ± 5.3
Dieldrin	12.4	75.5 ± 5.4	70.0 ± 6.4
o,p'-DDT	2.0	98.4 ± 10.1	96.4 ± 11.6
p,p'-DDT	8.3	84.2 ± 5.4	81.6 ± 7.0

[a] Mean percent ± SD in percent; $N = 5$ analytical runs of duplicates.

The recovery of *p,p'*-DDE is in contrast to data obtained in our laboratory when identical deproteinization techniques were used that resulted in higher yields (31). We believe that some losses probably occurred during the solvent evaporation and transfer that preceded the preparation of the spiking solution. Additional losses probably occurred during the sterile filtration.

Using analysis of variance, we evaluated the analytical data obtained for the CHs in TEPs 1–3 (Table 3). Generally, for the CHs the total coefficient of variation (CV) is higher in TEP 3 than it is in TEP 2. *o,p'*-DDT has the highest CV in TEP 2, and hexachlorobenzene has the highest CV in TEP 3.

A gas chromatogram of the reference standard AR 1254 is shown in Figure 4. The peaks are numbered relative to *p,p'*-DDE, which is assigned a relative retention time of 100. The gas chromatographic conditions were established so that the resulting gas chromatogram would be similar to that obtained by Webb and McCall (24) when they assigned their mean weight percent factors for the various peaks. Figures 4–6 demonstrate that, in general, most PCBs elute from the silica gel column in the second fraction. With the exceptions of *p,p'*-DDE 100 and *trans*-nonachlor 92, none of the CHs evaluated elute with the PCBs. These values, when compared with those shown in Figure 4 (standard), also demonstrate that bovines tend to retain to a larger extent those PCBs with relative retention times equal to or greater than 124. The data resulting from our characterization of the TEPs for PCBs are summarized in Table 4. The values reported are without the contribution of peak 98 (24). The contribution of this peak was not considered because of the variation in the ability of the adsorption chromatography to consis-

Table 3. Statistical analysis of characterized values obtained for TEPs (CH)

Pool	CH	Spike, ppb	*N*(runs)	Range, ppb	Mean, ppb	CV (%) for within-run component of variance	CV (%) for among-runs component of variance	CV (%) for within- and among-runs components of variance
TEP 1	*p,p'*-DDE[a]	—	5	0.46– 0.68	0.55	10.6	7.4	13.0
TEP 2	HCB	6.2	5	3.0– 4.1	3.6	*	*	8.6
	β-HCCH	8.4	5	4.3– 5.3	4.8	6.0	4.4	7.4
	oxychlordane	4.0	5	2.9– 3.4	3.1	3.1	4.4	5.4
	heptachlor epoxide	2.0	5	1.4– 1.9	1.6	5.0	5.4	7.4
	trans-nonachlor	4.8	5	4.1– 4.8	4.3	4.1	3.5	5.4
	p,p'-DDE	29.1	5	16.4–19.8	18.3	4.3	4.2	6.0
	dieldrin	12.4	5	8.2–10.4	9.3	4.2	6.3	7.6
	o,p'-DDT	2.0	5	1.7– 2.3	2.0	6.6	8.4	10.6
	p,p'-DDT	8.3	5	6.2– 7.9	7.0	5.8	3.0	6.6
TEP 3	HCB	6.2	5	2.4– 4.2	3.4	14.2	9.3	17.0
	β-HCCH	8.4	5	3.9– 4.9	4.4	5.8	3.8	7.0
	oxychlordane	4.0	5	2.7– 3.3	3.0	5.6	7.4	9.2
	heptachlor epoxide	2.0	5	1.3– 1.7	1.6	6.3	4.0	7.4
	trans-nonachlor	4.8	5	3.4– 4.6	4.1	8.0	4.8	9.4
	p,p'-DDE	29.1	5	16.2–20.5	18.2	6.8	5.3	8.6
	dieldrin	12.4	5	7.3– 9.8	8.6	*	*	9.2
	o,p'-DDT	2.0	5	1.6– 2.3	2.0	6.6	10.5	12.4
	p,p'-DDT	8.3	5	5.8– 7.5	6.8	6.8	5.2	8.6

[a] *p,p'*-DDE was the only chlorinated hydrocarbon found in TEP 1 that could be confirmed on 2 different columns by using electron capture detection.

* In the analysis of variance, if the within-run mean square value exceeds the among-runs mean square value, the within-run CV is set equal to the total CV and the among-runs CV is considered zero.

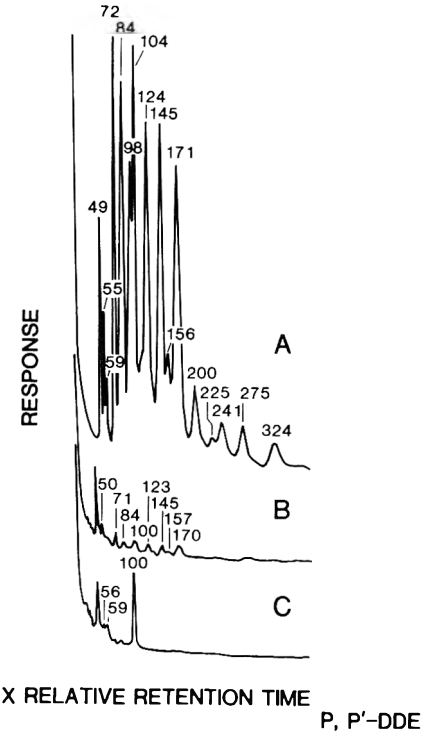

Figure 4. A, Aroclor 1254 standard (861 pg injected); B, TEP 1 Fraction II; C, TEP 1 Fraction III (12 mg injected, each fraction). For GLC conditions, see text.

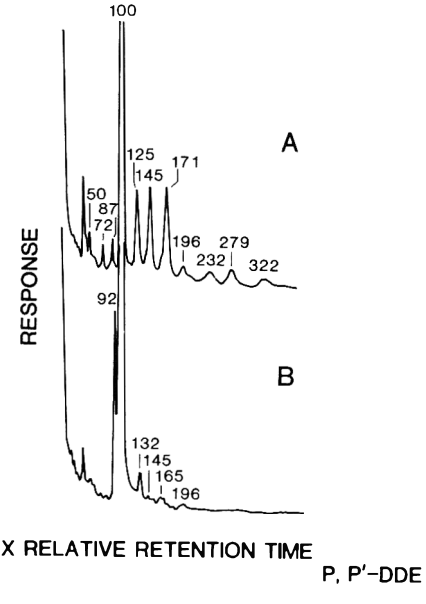

Figure 5. A, TEP 2 Fraction II; B, TEP 2 Fraction III (12 mg injected, each fraction).

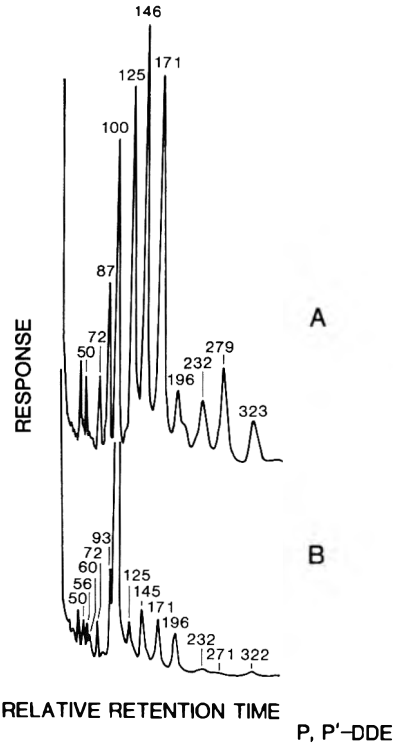

Figure 6. A, TEP 3 Fraction II; B, TEP 3 Fraction III (12 mg injected, each fraction).

tently preclude the elution of *p,p'*-DDE (at a retention time equivalent to peak 100) in fraction II. Because no effort was made to distinguish PCB peak 98 and *p,p'*-DDE, the contribution of peak 98 to the total was eliminated. Although the loss of peak 98 is not desirable from a methodology perspective, our experience in the analysis of general population samples and other investigators' experiences in the analysis of samples from individuals with known PCB exposure (32) indicate that peak 98 does not make a significant contribution to total in vivo PCB residues. Peak 98 in TEP 1 is not attributed to PCBs, but to *p,p'*-DDE; therefore, its elimination does not affect the PCB value of this pool. Based on the fortification levels of TEPs 2 and 3, the average recoveries were 114.1 and 92.6%, respectively, although the contribution of peak 98 was not considered.

The CVs for the PCB determinations were highest for the lowest concentration (TEP 2) and lowest for the highest concentration (TEP 3). These relationships are consistent with previous data generated by our laboratory.

The method presented here demonstrates some of the inherent problems associated with

Table 4. Statistical analysis of characterized values obtained for TEPs (PCBs)

Pool	Spike,[a] ppb	N(runs)	Range, ppb	Mean, ppb	CV (%) for within-run component of variance	CV (%) for among-runs component of variance	CV (%) for within- and among-runs components of variance
TEP 1	—	20	0.96– 4.41	2.38	15.6	29.2	33.1
TEP 2	9.97	20	7.70–16.27	11.38	8.4	15.4	17.6
TEP 3	49.64	20	31.49–54.42	46.01	6.4	9.6	11.5

[a] Defined as projected concentration resulting from dilution of serum taken from bovine exposed to Aroclor 1254 with PCB-free serum, i.e., bovine was exposed to AR 1254 and bled; PCB level was determined in serum, and serum was diluted.

concurrent determinations of intact exogenous compounds in serum. We believe that serum is the best possible matrix for the routine monitoring of such compounds. Although our analytical approach, which resulted in several fractions, did not completely separate the PCBs from the CHs, we believe that if one is to analyze samples containing a multitude of residues, then the procedures most likely will entail one of the following: (*1*) an analytical protocol similar to the one presented here, (*2*) a procedure that uses a series of different adsorbents, or (*3*) a procedure based on different activations or chemical treatments of a single adsorbent.

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Assessment of Methods to Determine PCB Levels in Blood Serum: Interlaboratory Study

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Twenty-five laboratories participated in a study to determine the levels of polychlorinated biphenyls (PCBs) in 3 bovine serum pools, referred to as trial environmental pools (TEPs). TEPs 2 and 3 contained, respectively, low (9.97 ppb) and high (49.64 ppb) levels of PCBs (as in vivo-bound Aroclor 1254) and the same level of 9 commonly occurring chlorinated hydrocarbons. TEP 1 contained only naturally occurring levels of these analytes. Laboratories analyzed each sample in duplicate by the method used in their laboratory for measuring PCBs in blood serum. The coefficients of variation (CV) for the 12 laboratories reporting quantitative data and the required number of analyses for TEP 2 and TEP 3 were 37.0 and 30.7%, respectively. The mean recoveries for these 12 laboratories were 239.3 and 165.4% for TEP 2 and TEP 3, respectively. Three laboratories reported data with mean values for TEP 2 and TEP 3 within ± 3 standard deviations of the CDC characterized mean. Their coefficients of variation were 12.4 and 18.8% for TEP 2 and TEP 3, respectively. The mean recoveries for these 3 laboratories were 150.7 and 98.4% for TEP 2 and TEP 3, respectively. Our most significant observations were the laboratories' failure to separate PCBs from DDTs and the excessive background of the reagent blanks. The widely discrepant results indicate a definite need to standardize methodology for this analysis.

Interlaboratory studies are widely used in evaluating methods for determining chlorinated hydrocarbons in whole blood (1), serum, and adipose tissue (2); polychlorinated biphenyls (PCBs) in chicken fat (3, 4), fish (3), and milk (4); and trace level hydrocarbons in mussels (5). We used an interlaboratory study to observe the state of the art for determining PCBs in blood serum. To this end, we solicited the participation of state, territorial, and private laboratories in analyzing bovine serum pools (6) for PCBs.

The purpose of this study was to assess the ability of laboratories to analyze serum accurately and precisely for PCBs in the presence of a selected number of chlorinated hydrocarbons, and, on the basis of the data generated, to initiate a step toward standardizing methods for determining PCBs in blood serum.

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Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

Protocol for Interlaboratory Study

Participants were supplied with 5 vials, each containing ca 9 mL pooled serum. Two vials were labeled trial environmental pool (TEP) 2, 2 were labeled TEP 3, and 1 was labeled TEP 1. Participants were also supplied with 1 vial of neat Aroclor (AR) 1254, Lot AK-38. They were requested to analyze each vial of serum in duplicate for PCBs by the method used in their laboratory and to quantitate the PCBs by using the appropriate standards prepared from the Aroclor reference material. Participants were informed that the pools were prepared from bovine serum that contained both in vivo-bound PCBs (as AR 1254, Lot AK-38) and in vitro-fortified levels of chlorinated hydrocarbons.

Although no restrictions were placed on the analytical methods used, participants were asked to report the following:

1. Extraction: solvents and extraction techniques used
2. Cleanup procedures, if any: adsorbents used, eluting solvents and volumes, and liquid partitioning
3. Gas chromatography: column dimensions, column packing, pertinent parameters, and detector type (including operational parameters)
4. Method of quantitation: summation technique, e.g., the total area or total peak height of PCBs in the sample compared with the total area or total peak height in the Aroclor reference, excluding chlorinated hydrocarbon interference; the Webb and McCall approach (7); or the mean response of a selected number of peaks

Results and Discussion

Our approach to the separation and quantitation of PCBs contained in trial environmental pools has been reported (6).

We asked 54 state and territorial health laboratories and 117 private laboratories to participate

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in the interlaboratory study; 20 (37%) state laboratories and 5 (4%) private laboratories reported analytical data. One federal agency participated; for purposes of this study, that laboratory was considered a state participant.

Because all reporting laboratories had analyzed these samples by electron capture gas-liquid chromatography (ECGLC), as we had done, we invited participation from laboratories reporting in the scientific literature that they had determined PCB levels by other methods: glass capillary gas chromatography with electron capture detection, radioimmunoassay, radioisotope dilution assay, and gas chromatography/mass spectrometry (GC/MS). These laboratories, however, did not have experience in determining PCBs at the low levels found in serum and thus encountered many problems. All of these methods except GC/MS were abandoned. The laboratory using this technique had no experience in the extraction and cleanup of PCBs in serum; therefore, we performed these steps by using the method previously presented (6), and forwarded the column eluates for GC/MS analysis. The laboratory monitored 6 ions by GC/MS and reported the levels of tetrachlorobiphenyl, pentachlorobiphenyl, and hexachlorobiphenyl found (Table 1). These results compare well with ours (6); however, because this laboratory did not perform the analysis in its entirety, its results are not included in this report. These limited data tend to confirm our quantitative technique but, of course, do not indicate the efficiency of our extraction or cleanup steps.

We had thought that vials containing 9 mL serum would be more than adequate for duplicate analysis; however, some participants used serum aliquots of 5–6 mL, which precluded duplicate analysis from the same vial. Summaries of the significant parameters and analytical results are presented in Tables 2 and 3, respectively.

Extraction techniques used by participating laboratories followed 2 general approaches: direct extraction or extraction following deproteinization with methanol. Nine (36%) of the laboratories used methanol for deproteinization, followed by extraction with an immiscible organic solvent; 12 (48%) extracted directly with an immiscible organic solvent; 2 (8%) extracted with acetonitrile, added an aqueous salt solution, and partitioned into hexane or petroleum ether; one (4%) adsorbed the serum onto Florisil; and one (4%) did not report the extraction technique used. Our experience has shown that deproteinization of the blood serum with methanol (8) enhances the recovery of halogenated biphenyls.

Table 1. Results [a] (ppb) of gas chromatography/mass spectrometry (GC/MS) analysis of extracts of trial environmental pools (TEPs)

Pool No.	Cl_4	Cl_5	Cl_6	Total AR 1254
TEP 1	1.6	1.5	0.9	4
TEP 2	2.5	6.9	4.8	14
TEP 3	5.2	22.1	20.8	48

[a] Mean of 2 GC/MS analyses per pool.

Most laboratories used the more commonly available adsorbents for cleanup, with the exception of alumina. Silica gel, silicic acid, and Florisil were used almost equally. One laboratory used 2 adsorbents, Florisil and silica gel, and 4 laboratories chose not to clean up their extracts. Florisil does not separate chlorinated hydrocarbons from PCBs as completely as does silica gel (9, 10). Silica gel has been treated in various ways to achieve this separation (11–13). Although blood serum does not present as extensive a cleanup problem as some other matrices, the presence of multiple residues and the fouling effect of continuous injections of small amounts of lipids into the gas chromatograph/electron capture detection system dictate the use of adsorption chromatography or some form of cleanup before ECGLC.

For the elution of PCBs from adsorption columns, most laboratories used nonpolar solvents. In a very few instances, small amounts of more polar solvents, namely, methanol and ethyl ether, were mixed with nonpolar solvents for eluants. The elution volumes were generally excessive, even though some of the adsorption columns used would be considered small ($\leq$10 mm id). We have found that blood serum can be adequately cleaned up with quantitative recovery of chlorinated hydrocarbons and PCBs by using adsorption columns <10 mm id with solvent elution volumes <50 mL. Use of too much solvent tends to produce reagent blanks that approach the lower concentration levels of blood serum for some halogenated compounds, i.e., <10 ppb.

Only 2 laboratories used chemical treatment in addition to adsorption chromatography to prevent the interference of chlorinated hydrocarbons in determining PCBs. The 2 chemical treatment procedures used have certain disadvantages: perchlorination of the PCBs to decachlorobiphenyl destroys the identity of PCB

Table 2. Significant analytical variables used by participating laboratories[a]

Lab.	Serum Vol. (mL)	Extraction technique: Deprotein-ization with methanol	Extraction technique: Extraction solvent(s)	Method of cleanup		Elution (for PCB only): Vol. (mL)	Elution (for PCB only): Eluant	GC Column type	Quantitation method (see text)
S-1	5	yes	hex/Et_2O	SA CC-4	M	175	pet et	NP	peak height
S-2	2	no	hex	SA (3)	M	250	pet et	MP	disc integrator
S-3	2	no	hex	F	M	200	pet et	NP	peak height
S-4	6	no	hex	none				MP	peak height
S-5	5	yes	hex/Et_2O	SG (1)	μ	8	hex	MP	elect. integrator
S-6	5	yes	hex/Et_2O	SA CC-4	μ	170	pet et	MP	peak height
S-7	2	no	hex	none				MP	peak height
S-8	NR	no	acn; pet et	F	M	150	pet et	NP	peak height
S-9	2	no	hex	none				MP	peak height
S-10	4	yes	hex/Et_2O	SG (3)	μ	25	hex	MP	peak height
S-11	5	yes	Et_2O/pet et	F; SG (3)	M	18; 18	pet et; hex	NR	perchlorination
S-12	4	yes	hex/Et_2O	SG	μ	30	hex	NP	integrator (W&Mc)
S-13	NR	NR	NR	F	NR	200	hex	MP	integrator
S-14	2	no	acn; hex	F	M	250	6% Et_2O:hex	MP	peak height
S-15	2	no	hex	SG	μ	85	hex	P	peak height
S-16	4	yes	hex/Et_2O	SG (3)	μ	20	hex	NP	elect. integrator
S-17	2	no	hex	SA CC-4	μ	25	pet et	MP	peak height
S-18	3	no	adsorbed on F	F sapon. & oxid.	M	200	pet et	MP	integrator (W&Mc)
S-19	2	no	hex	SA CC-4	μ	120	pet et	MP	peak height
S-20	2	no	hex	F	M	250	pet et	P	peak height
P-1	4	no	hex	SG	μ	30	pet et	NP	peak height
P-2	NR	no	hex	SG	μ	30	pet et	NP	peak height
P-3	5	yes	hex/Et_2O	F	μ	14	1.0% MeOH:hex	NP	peak height
P-4	2	yes	pet et	none				NP	peak height
P-5	NR	no	isop/pet et	F	NR	NR	NR	NP	peak height

[a] Legend:

NR	not reported	F	Florisil	P	polar: OV-210
hex	hexane	SA CC-4	silicic acid (Cromartie, E., et al. (1975) *Pestic. Monit. J.* **9**, 11–14)	NP	nonpolar: OV-101, SE-30, SP2100, OV-1
Et_2O	ethyl ether				
pet et	petroleum ether	SA	silicic acid (*PAM* Vol. 1, Sec. 251.1)	MP	mixed phases SE-30/OV-210, OV-17/QF-1, OV-1/OV-210, OV-1/QF-1, OV-17/OV-210, OV-101/OV-210
acn	acetonitrile				
isop	isopropanol	M	macro if adsorption column id > 10 mm	W&Mc	Webb & McCall (7)
SG	silica gel				
( )	% deactivation with water	μ	micro if adsorption column id, <10 mm		

Table 3. Participating laboratories' reported levels (ppb) of polychlorinated biphenyls in trial environmental pools (TEPs)[a]

	TEP 1		TEP 2				TEP 3			
	Vial 1		Vial 1		Vial 2		Vial 1		Vial 2	
Lab.	Orig.	Dupl.	Orig.	Dupl.	Orig.	Dupl.	Orig.	Dupl.	Orig.	Dupl.
S-1	ND	NA	15.7	17.5	18.5	24.3	90	109.8	80	70
S-2	59.3	64.8	0	NA	0	NA	94.4	NA	97.3	NA
S-3	ND	NA	10	NA	10	NA	22	NA	39	NA
S-4	ND	NA	62.1	NA	68.7	NA	100.5	NA	134.8	NA
S-5	ND	NA	12	NA	14	NA	43	NA	46	NA
S-6	2	1	17	20	19	19	88	96	75	82
S-7	ND	ND	ND	ND	ND	ND	37	47.5	45.1	45.2
S-8	16	NA	14	NA	28	NA	22	NA	41	NA
S-9	<10	<10	23.3	23.6	23.1	22.2	46.7	43.0	40.4	42.0
S-10	6.9	NA	25	NA	18	NA	38	NA	NA	NA
S-11	4.3	NA	27.2	NA	NA	NA	95.2	NA	NA	NA
S-12	2.0	1.5	14.6	16.7	16.4	16.4	57.9	56.3	59.8	56.3
S-13	<10	27	34	26	28	32	90	85	88	102
S-14	ND	ND	ND	ND	ND	ND	27.5	21	18	19
S-15	21	32	28	39	54	34	64	87	96	74
S-16	ND	NA	15.3	18.9	13.8	14.3	41.9	51.0	33.2	42.9
S-17	2.3	1.9	20.1	20.6	NA	NA	51.7	60.4	ND	NA
S-18	ND	NA	14	13	14	13	50	38[b]	50	49
S-19	<5	6.4	28.8	30.4	30.3	31.8	94.7	90	90.8	96.5
S-20	0	0	32.6	37.4	NA	NA	71.3	65.3	57.4	56.1
P-1	0	11	35	30	29	37	105	98	99	113
P-2	0	9	25	29	30	35	90	93	91	98
P-3	ND	ND	19.3	20.7	17.6	17.9	78.8	88.8	92	98
P-4	37.1	NA	52.9	NA	36.2	NA	90.3	NA	103	NA
P-5	<5	<5	20	20	NA	NA	40	50	NA	NA
Fortification level	—		9.97				49.64			
CDC value	2.38 ± 0.79		11.38 ± 2.00				49.06 ± 5.30			

[a] ND = Not detectable at the participants' lower detection limits; NA = not analyzed.
[b] Triangulation method, electronic integrator inoperable.

species, and alkaline hydrolysis followed by chromic acid oxidation of the commonly occurring chlorinated hydrocarbons reportedly results in the loss of certain PCBs (14). On the other hand, the latter procedure has also been reported to effectively recover and separate PCBs, although this was accomplished at PCB levels of tenths (15) or tens (16) of micrograms.

All laboratories used ECGLC for analysis, and all but one used the ^{63}Ni detector. Only column selection for quantitative runs is reported in Table 2; however, some participants used 2 or more columns for confirming PCBs. Choices between mixed and nonpolar phases were 48% and 40%, respectively. Only 2 participants chose a polar phase for quantitation. One laboratory did not report the type of GC column used. We used GC columns with the methyl silicones as liquid phases, mainly because they had been chosen for another project (17) and also because we intended to use the Webb and McCall approach to quantitate PCBs (7).

Most of the laboratories used peak heights to measure in vivo PCBs. The retention times of PCBs in the reference material were matched to peaks observed in the samples. The number of peaks selected varied from 2 to 11. Peaks were manually measured in most instances. One participant used a disc integrator and 5 others used electronic integration to assess peak areas. Only 2 laboratories used the Webb and McCall approach. We have found that when there is no great difference between the in vivo PCB patterns and those of an appropriate PCB standard, analyte values obtained by use of the Webb and McCall method do not differ appreciably from values obtained by summation of peak heights.

This study substantiates the difficulty associated with determining PCBs in the presence of chlorinated hydrocarbons in blood serum. TEPs 2 and 3 represent some of the most extreme situations that a chemist is likely to encounter in analyzing blood serum, because—with the exception of the DDTs (*p,p'*-DDE, *o,p'*-DDT, and *p,p'*-DDT)—the chlorinated hydrocarbons that were added to these pools appear in human serum in various frequencies and at relatively low mean concentrations, i.e., β-HCCH (27%, 0.6 ppb), dieldrin (13%, 2.4 ppb), heptachlor epoxide (1.3%, 0.02 ppb), oxychlordane (3.9%, 0.06 ppb),

Table 4. Statistical analysis of participating laboratories' (PCB) results for trial environmental pools (TEPs) 2 and 3

Pool ID	FL[a] (ppb)	N (Labs)	Range of values	Mean (ppb)	CV (%) for within-lab. component of variance	CV (%) for among-labs component of variance	CV (%) for within- and among labs components of variance
TEP 2	9.97	12[b]	13.00–54.00	23.86	16.8	33.0	37.0
TEP 3	49.64	12[b]	33.20–113.00	76.08	11.0	28.6	30.7
TEP 2	9.97	3[c]	13.00–18.90	15.03	9.8	7.4	12.4
TEP 3	49.64	3[c]	33.20–59.80	48.86	11.2	15.1	18.8

[a] FL = Fortification level defined as projected concentration resulting from dilution of serum taken from bovine exposed to Aroclor 1254 with PCB-free serum, i.e., bovine was exposed to AR 1254 and bled; PCB level determined in serum and serum diluted.

[b] Participants that performed duplicate analyses of both vials from TEPs 2 and 3 and obtained quantitative data.

[c] Participants that met the criteria in *b* and also had mean values for TEP 2 and 3 that were within ± 3 standard deviations of the CDC characterized mean.

trans-nonachlor (12.4%, 0.20 ppb), and HCB (2.9%, 0.06 ppb). The in vitro spiking levels we used approach the maximum storage levels in serum reported by the Environmental Protection Agency for all compounds except the DDTs, which approach the reported mean storage levels in serum. Usually chemists can expect to encounter the DDTs, which are almost ubiquitous.

The in vivo-bound PCBs (as AR 1254) are predominantly composed of isomers that elute after *p,p'*-DDE; consequently, if the PCBs and the DDTs can be separated, PCBs can be determined. In this study, most of the laboratories quantitated the samples on the basis of peaks eluting after *p,p'*-DDE. Because no restrictions were placed on the analytical methods used by participants, many variables could have contributed to the unusually high levels reported; however, our review of the analytical procedures and the gas chromatograms submitted indicated that the wide discrepancies could be attributed essentially to 1 or 2 variables or a combination of both. The first was a failure to completely separate PCBs from DDTs. The second was the presence of unusually high reagent blanks, caused partly by using too much solvent. In view of this, it is interesting to note (Table 3) that of those laboratories (S-12, S-16, and S-18) that reported data with a mean value for duplicates within ± 3 standard deviations of the CDC mean for both augmented pools, 2 used silica gel with a minimum amount of solvent for elution and the third (S-18) used Florisil followed by saponification and acid oxidation. This reaffirms the need to prevent interference from DDTs.

Using analysis of variance, we evaluated the data submitted by those participants that adhered to protocol and obtained quantitative data for TEPs 2 and 3 (Table 4). The intralaboratory coefficient of variation (CV) is lower than the interlaboratory or total CV. However, the mean values of 23.86 and 76.08 ppb represent 239.3 and 165.4% recovery based on the fortification levels of TEP 2 and 3, respectively. We repeated the analysis of variance using only data submitted by participants whose mean values for TEP 2 and 3 were within ± 3 standard deviations of the CDC characterized mean (Table 4). The resulting coefficients of variation are lower and more compatible with the CDC data (6). The mean values of 15.03 and 48.86 represent 150.7 and 98.4% recovery based on the fortification levels of TEP 2 and 3, respectively. These recoveries are higher, especially TEP 2, than the recovery obtained by CDC (6).

This report demonstrates the need for improving analytical methods for determining PCBs in blood serum. Because blood serum has been and will probably continue to be the matrix of choice for monitoring PCB levels in humans and PCBs undoubtedly will continue to be a nemesis for residue chemists, methodological guidelines should be established for this type of analysis.

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VITAMINS AND OTHER NUTRIENTS

Sources of Variance in the Bioassay of Protein Value

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A factorial design was used to simultaneously evaluate the relative effect of 8 experimental factors and their interactions on the weanling rat bioassay of protein value: (*1*) source of protein: ANRC casein, lactalbumin, high-protein wheat flour; (*2*) protein level of the diet: 5 and 10%; (*3*) dietary fat level: 10 and 20% corn oil; (*4*) animal: ARS-Sprague-Dawley, from Taconic Farms; (*5*) age of animal: 21 and 28 days; (*6*) acclimation time: 2 and 4 days; (*7*) replication: 2 complete replications in time; and (*8*) duration of the test: food consumption and body weights were measured at 3, 7, 10, 14, 17, 21, 24, and 28 days after starting the test diet and converted to the ratio of grams of weight gained per gram of protein consumed for each weigh day. The official AOAC method for determining the protein efficiency ratio was followed with minor modifications. All 8 factors and many of their possible interactions appeared to significantly influence the measured ratios. When the ratios were adjusted by reference to the corresponding value for casein as a control, fat level, rat source, rat age, and acclimation time were no longer significant sources of variation. Plots of measured ratios and their coefficients of variation against time suggest that the optimum assay time varies with the protein but that the assay time should not be less than 21 days. The generally used 28-day assay time seems to offer no increase in precision over 21 days.

The various animal assays that have been used to measure protein quality have been reviewed extensively (1–5). The protein efficiency ratio (PER) method has received wide usage and official recognition in the United States and Canada through its incorporation in *Official Methods of Analysis* of the AOAC (6). This method has the serious disadvantage of being suitable only for products of high protein content, such as supplements and concentrates, for which it was originally developed. Specific limitations of the PER assay are as follows: the results are positively correlated to food intake and to weight gain (7, 8); it makes no allowance for the maintenance requirements of the rat (7); the response is not linear (8); the responses for different proteins are not parallel (8); comparisons are made at equal doses of the test protein rather than at equal responses (8); and the level of protein used in the diet (10%) is biased against most plant sources of protein (9).

Regardless of the animal assay used, a number of factors influence the results obtained. Although these factors have been studied the most extensively for the PER test, they can affect other animal assays as well. The weight gain per unit of protein consumed is influenced by the strain of the rat (10–12), sex of the rat (10), age of the rat at the beginning of the test (13), size of the litter from which the rat came (14), acclimation period (15), length of the experimental period (10–13), level of dietary protein (10, 13, 16), type of dietary carbohydrate (17–19), level of fiber in the diet (12), level of moisture in the diet (15, 20–22), and the manner in which water is added to the diet (22). Some experiments have indicated that factors such as the fat level of the diet and the mineral mixture employed have no significant effect (12, 15, 16); other experiments have shown that use of a reference casein as an internal standard largely eliminates differences due to the strain of the rat and the fiber and moisture content of the diet (10–12).

The AOAC test protocol (6, secs. **43.212-43.216**) for measuring protein quality is written to permit the analyst to vary experimental parameters in ways which may affect the reproducibility of the test results. Although individual laboratories may adhere to fixed protocols permitted within the broad limits of the published method, it is probable that marked deviations between laboratories exist without being recognized or even specified when data are reported.

The study reported here grew out of the experiences and needs of a Committee of the Division of Agricultural and Food Chemistry (AGFD) of the American Chemical Society formed to serve in a liaison capacity between the League for International Food Education (L.I.F.E.) and the membership of AGFD. From 13 factors identified as having the potential to influence the observed growth response of the

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rat to the administered protein, the AGFD/L.I.F.E. Committee chose the following 8 for study.

(1) *Source of Protein.*—Lactalbumin, casein, and a high-protein wheat flour were selected to provide a wide range of protein quality over which to check the other factors and to provide a measure of the method sensitivity.

(2) *Protein Level.*—Two levels, 5 and 10% protein (N × 6.25), were selected. The 10% protein level is commonly used in the AOAC PER method. The 5% level was chosen as a low-protein level at which only rats fed high-quality proteins will gain weight.

(3) *Fat Level.*—Two levels of refined corn oil, 10 and 20%, were chosen to represent 4 distinct protein/calorie ratios.

(4) *Source of Animal.*—Animals from 2 major commercial breeders of albino rats were used in the study.

(5) *Age of Rat.*—The 2 extremes in rat age (21 and 28 days) permitted by the AOAC PER method were chosen for this factor.

(6) *Acclimation Period.*—The 2 extremes in acclimation period (2 and 4 days) permitted by the AOAC PER method were chosen. A stock laboratory maintenance diet was selected for the acclimation period, with the understanding that this factor could interact with the protein under test.

(7) *Time of Performance.*—This factor was essentially a replication in time, used to maintain more uniform conditions within the treatment combinations as well as to eliminate possible irrelevant sources of variation among the treatment combinations.

(8) *Test Period.*—Food consumption and animal weight were measured on days 3, 7, 10, 14, 17, 21, 24, and 28 after the start of the test diet, providing an insight into this factor and a check on whether the animals were within the linear growth phase.

The objective of this study was to identify, among selected factors, those most affecting the precision of the measurement of protein value. A protocol was developed by the AGFD/L.I.F.E. Committee and the study was performed under contract to L.I.F.E. by Technological Resources, Inc., a service subsidiary of Campbell Soup Co., Camden, NJ. This is a report of that evaluation with additional statistical analysis.

Experimental

Experimental Design

The full factorial design contained 96 cells (5 rats/cell) and was run in full in 2 replications in time for a total of 960 rats as shown below:

Factorial Design:

$3 \times 2 \times 2 \times 2 \times 2 \times 2 \times 2 = 192$ cells

5 rats per cell × 192 cells = 960 rats

Variables:

Protein source: casein, lactalbumin, wheat flour

Protein level: 5%, 10%

Fat level: 10%, 20%

Animal source: ARS-Sprague-Dawley, Taconic Farms

Age of rat: 21 days, 28 days

Acclimation period: 2 days, 4 days

Replication: replication time 1, replication time 2

Measurements:

Food consumption at 3, 7, 10, 14, 17, 21, 24, 28 days

Body weights at 0, 3, 7, 10, 14, 17, 21, 24, 28 days

A design of this magnitude was imposed to more fully evaluate the relative effects of the various selective factors known to influence protein value, both separately and in combination, in a single study.

Animal Facility

The rats were individually housed in raised stainless steel cages in a room with controlled temperature (22–24°C), humidity (50–55% relative humidity), and lighting (100 ft-candles at median cage level controlled by timer for 12 h light and 12 h dark). The animals had free access to food and water at all times. Water bottles were checked daily and changed a minimum of twice weekly. Food was provided fresh on each weigh day in food cups with anti-scatter rings. Animal housing, diet preparation, and the food cup filling and weighing operations were each performed in separate rooms. These rooms were kept free of insects and rodents by stringent housekeeping and regular pest control.

Animal Procurement, Acclimation, and Randomization

Male Sprague-Dawley rats, 21 or 28 days of age, were purchased from ARS-Sprague-Dawley, Madison, WI, and from Taconic Farms, Germantown, NY.

Rats in each age category were caged singly on arrival and acclimated on Purina Laboratory Chow for 2 or 4 days before assignment to the experimental diets.

Following acclimation, the rats were placed in randomly placed rack positions using tables of random numbers. Some rats at the extremes of

Table 1. Composition (%) of protein sources

Protein source	Moisture	Protein[a]	Fat	Carbohydrate[b]	Ash
ANRC casein[c]	5.4	87.8	1.2	4.5	1.1
Lactalbumin[d]	4.1	79.9	7.0	6.1	2.9
Wheat flour[e]	11.3	15.5	71.2	1.5	0.5

[a] Protein = N × 6.25.
[b] By difference.
[c] Animal Nutrition Research Council casein obtained from Humko Sheffield Chemical Co., Memphis, TN.
[d] Lactalbumin No. 8NC15 obtained from Humko Sheffield Chemical Co.
[e] High-protein wheat flour supplied by Pillsbury Co., Minneapolis, MN.

the weight distribution curves were discarded in an attempt to ensure that the average weights of all groups having the same age rats and the same acclimation period were within a 6 g range and the weight range of individual rats within a group did not exceed 10 g.

Preparation of Diets

The composition of the 3 protein sources used in the diets is shown in Table 1.

The 12 diets were formulated in accordance with the general procedures specified in the AOAC PER determination (6, secs. **43.212–43.216**) with the following modifications: USP XVIII salt mixture was replaced with an equal amount of a Bernhart and Tomarelli salt mixture (23), the balancing carbohydrate was food-grade corn starch, and no adjustment was made in the moisture level because both flour and corn starch normally contain 10–12% moisture at equilibrium under ambient conditions. The diet formulations are given in Table 2. Each diet was made in sufficient quantity to last the entire study, and was stored in air-tight containers at 4°C until used. Five random samples of each diet were analyzed for proximate composition by AOAC official methods (6) for moisture in sugars (sec. **31.005**) to determine total solids; for fat in animal feed (secs. **7.055–7.056**); for ash in cereal foods (sec. **14.006**); and for nitrogen in fertilizers (sec. **2.058**).

Data Acquisition and Analysis

During the course of both replicates, animal conditions, animal weights, full food cup weights, and empty food cup weights were recorded using a Mettler PT15 electronic balance interfaced to a Model 33 teletype with paper tape punch. The paper tapes were read into a time-shared computer for processing using Fortran programs written for PER calculations. The rats were weighed on receipt from the breeder; on

Table 2. Diet formulations

Test diets	Diet ingredients, %					
	Protein source[a]	Corn starch[b]	Corn oil[c]	Mineral mix[d]	Cellulose[e]	Vitamin mix[f]
5% ANRC casein—10% fat	5.69	78.44	9.93	3.94	1.00	1.00
5% Lactalbumin—10% fat	6.26	78.30	9.62	3.82	1.00	1.00
5% Wheat flour—10% fat	32.36	52.38	9.52	3.84	1.00	1.00
5% ANRC casein—20% fat	5.69	68.44	19.93	3.94	1.00	1.00
5% Lactalbumin—20% fat	6.26	68.30	19.62	3.82	1.00	1.00
5% Wheat flour—20% fat	32.26	43.38	19.52	3.84	1.00	1.00
10% ANRC casein—10% fat	11.39	72.88	9.86	3.87	1.00	1.00
10% Lactalbumin—10% fat	12.41	72.26	9.52	3.81	1.00	1.00
10% Wheat flour—10% fat	64.94	20.48	8.90	3.68	1.00	1.00
10% ANRC casein—20% fat	11.39	62.88	19.86	3.87	1.00	1.00
10% Lactalbumin—20% fat	12.41	62.26	19.52	3.81	1.00	1.00
10% Wheat flour—20% fat	64.94	10.48	18.90	3.68	1.00	1.00

[a] ANRC casein and lactalbumin No. 8NC15 supplied by Humko Sheffield Chemical Co.; high-protein wheat flour supplied by Pillsbury Co.
[b] USP grade corn starch supplied by Bio Serv, Inc., Frenchtown, NJ.
[c] Mazola corn oil supplied by Best Foods, a division of CPC International, Corpus Christi, TX.
[d] Bernhart-Tomarelli salt mixture supplied by Bio Serv, Inc.
[e] Cellulose (BW 200) supplied by Bio Serv, Inc.
[f] AOAC vitamin mixture supplied by Bio Serv, Inc.

Table 3. Proximate composition (%) of test diets[a]

Test diets	Solids		Carbohydrate		Fat		Protein (N × 6.25)		Ash	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
5% Casein—10% fat	91.6	0.3	71.9	0.8	9.9	0.3	5.8	0.6	3.7	0.2
5% Casein—20% fat	92.4	0.1	62.6	0.3	19.9	0.3	6.1	0.2	3.9	0.1
10% Casein—10% fat	93.5	0.7	69.6	0.8	9.9	0.2	10.1	0.2	3.9	0.1
10% Casein—20% fat	94.7	0.4	60.4	0.4	20.0	0.1	10.3	0.1	4.1	0.1
5% Lactalbumin—10% fat	91.7	0.5	71.4	2.0	10.6	1.8	5.9	0.4	3.8	0.1
5% Lactalbumin—20% fat	93.7	0.4	64.7	0.4	19.6	0.1	5.4	0.1	4.0	0.1
10% Lactalbumin—10% fat	93.7	0.8	69.3	0.9	10.1	0.2	10.3	0.1	3.9	0.1
10% Lactalbumin—20% fat	93.7	0.4	59.4	0.4	19.9	0.1	10.4	0.2	3.9	0.0
5% Wheat flour—10% fat	91.9	0.5	72.9	0.4	10.0	0.2	5.2	0.1	3.9	0.0
5% Wheat flour—20% fat	93.0	0.4	64.3	0.6	19.6	0.3	5.2	0.1	3.9	0.0
10% Wheat flour—10% fat	91.9	0.3	68.4	0.2	9.5	0.1	10.1	0.1	3.9	0.0
10% Wheat flour—20% fat	92.3	0.6	58.6	0.5	19.7	0.0	10.1	0.2	3.9	0.1

[a] Mean and standard deviation of 5 determinations for each analysis.

the last day of the acclimation period; and on days 3, 7, 10, 14, 17, 21, 24, and 28 during the 4-week test period. The food was weighed and replaced on each weigh day.

Food consumption and weight gain data were available for 3, 7, 10, 14, 17, 21, 24, and 28 days. The weight gain/protein consumption ratios were calculated for all weigh days; ratios compared to casein were calculated for days 7, 14, 21, and 28.

The unadjusted gain/consumption ratios for casein, lactalbumin, and wheat flour and the ratios for lactalbumin and wheat flour compared with casein at days 14, 21, and 28 were analyzed by 5-way analysis of variance. In these analyses, the replication factor was treated as a random rather than a fixed factor (24).

The unadjusted ratios and the coefficients of variation (CV) of the ratios for each protein at each level were pooled and plotted vs time on diet to determine the dependence of the magnitude and precision of the protein quality measurement on that variable.

Results and Discussion

The proximate analyses of the diets (Table 3) show that the diets were close in composition to the formulations. Three of the 5% protein diets (5% casein–10% fat, 5% casein–20% fat, and 5% lactalbumin–10% fat) were outside the ±0.5% protein specification of the protocol due to the high level of corn starch in these diets. The corn starch used contained 0.3% protein. The targeted percentage of fat (10 or 20%) was achieved within an acceptable range (e.g., 9.5–10.6% for 10% fat and 19.6–20% for 20% fat). Water was not added to the diets except for that present in the wheat flour and corn starch; the moisture content of the diets ranged from 5.3 to 8.4%. This small difference in moisture content is not likely to influence food consumption.

After randomization, the average group weights for the same age rats and the same adaptation period were nearly the same regardless of supplier and replicate. However, the 28-day-old rats from both suppliers after 4 days of adaptation (28-4 group) exceeded the 10 g maximum spread within-group requirement in both the first and second replicates. In addition, the Taconic Farms 28-2 and 21-4 groups exceeded the within-group limit. Not only is it difficult to buy 28-day-old rats in a narrow weight range, but after 4 days of adaptation, the weight range has widened so that the spread within groups exceeds the 10 g specified by the AOAC procedure (6). No problem was encountered in meeting

Table 4. Twenty-eight-day weight gain, g [a]

Protein source	Rat age, days	Replicate	5% Protein				10% Protein			
			AP1 (2)		AP2 (4)		AP1 (2)		AP2 (4)	
			SD	TF	SD	TF	SD	TF	SD	TF
10% Fat Level										
Casein	21	1	23	23	20	28	64	81	72	103
		2	18	31	16	21	95	104	82	106
	28	1	11	2	17	0	81	86	56	67
		2	4	13	11	3	74	72	71	86
Lactalbumin	21	1	54	59	42	42	94	93	102	86
		2	44	58	48	70	122	115	114	121
	28	1	43	44	22	28	75	68	57	75
		2	38	41	28	40	82	149	112	115
Wheat flour	21	1	−5	−7	14	−3	12	7	8	14
		2	−4	−5	−6	−5	0	12	8	11
	28	1	−11	−14	−4	−8	6	5	12	6
		2	−16	−13	−13	−16	9	−3	13	5
20% Fat Level										
Casein	21	1	18	17	11	20	70	70	63	83
		2	13	22	2	20	66	80	65	70
	28	1	3	−2	9	4	59	44	58	72
		2	4	−1	1	0	63	56	78	77
Lactalbumin	21	1	40	46	36	47	129	106	97	113
		2	41	51	28	55	134	149	131	147
	28	1	33	36	34	30	117	133	113	105
		2	41	34	49	36	152	132	155	147
Wheat flour	21	1	−4	−4	−5	−7	8	23	5	7
		2	−5	−5	−10	−5	7	6	6	9
	28	1	−13	−14	−17	−14	3	7	7	7
		2	−13	−14	−15	−19	4	−1	11	−1

[a] SD = Sprague-Dawley, TF = Taconic Farms, AP1 (2) = 2-day acclimation, AP2 (4) = 4-day acclimation.

Table 5. Twenty-eight-day weight gain/protein consumption ratio [a]

Protein source	Rat age, days	Replicate	5% Protein				10% Protein			
			AP1 (2)		AP2 (4)		AP1 (2)		AP2 (4)	
			SD	TF	SD	TF	SD	TF	SD	TF
10% Fat Level										
Casein	21	1	1.50	1.62	1.16	1.75	1.71	2.32	2.10	2.69
		2	0.80	1.56	0.84	1.08	2.19	2.59	1.84	2.67
	28	1	0.65	0.08	0.81	−0.07	2.14	2.32	1.40	1.78
		2	0.20	0.56	0.43	0.18	1.57	1.65	1.70	1.83
Lactalbumin	21	1	2.71	2.90	1.95	2.07	2.70	2.75	2.77	2.62
		2	1.74	2.54	1.90	2.88	2.46	2.42	2.34	2.69
	28	1	2.00	1.99	1.03	0.96	2.11	1.95	1.67	2.02
		2	1.46	1.75	1.08	1.54	1.69	2.96	2.34	2.41
Wheat flour	21	1	−0.48	−0.78	1.25	−0.39	0.52	0.36	0.38	0.61
		2	−0.25	−0.41	−0.38	−0.35	0.25	0.47	0.28	0.35
	28	1	−0.95	−1.35	−0.34	−0.64	0.23	0.21	0.40	0.23
		2	−0.91	−0.85	−0.76	−0.93	0.24	−0.07	0.29	0.16
20% Fat Level										
Casein	21	1	1.20	1.42	0.84	1.38	2.23	2.35	2.31	2.88
		2	0.81	0.81	0.15	0.15	1.62	1.99	1.53	1.79
	28	1	0.20	−0.19	0.59	0.24	1.77	1.21	1.90	1.93
		2	0.20	0	0.06	−0.05	1.06	1.14	1.81	1.58
Lactalbumin	21	1	2.74	3.29	2.12	2.91	3.36	2.77	2.39	2.95
		2	2.21	3.14	1.27	2.79	2.81	3.33	2.91	3.22
	28	1	1.77	2.13	1.77	1.77	2.70	3.27	2.45	2.48
		2	1.64	1.66	1.95	1.75	3.05	2.63	2.90	2.90
Wheat flour	21	1	−0.54	−0.54	−0.52	−0.70	0.43	1.19	0.26	0.39
		2	−0.47	−0.47	−0.55	−0.53	0.18	0.34	0.91	0.40
	28	1	−1.33	−1.39	−1.53	−1.23	0.13	0.26	0.29	0.22
		2	−0.97	−0.87	−0.88	−1.19	0.14	−0.04	0.34	−0.07

[a] SD = Sprague-Dawley, TF = Taconic Farms, AP1 (2) = 2-day acclimation, AP2 (4) = 4-day acclimation.

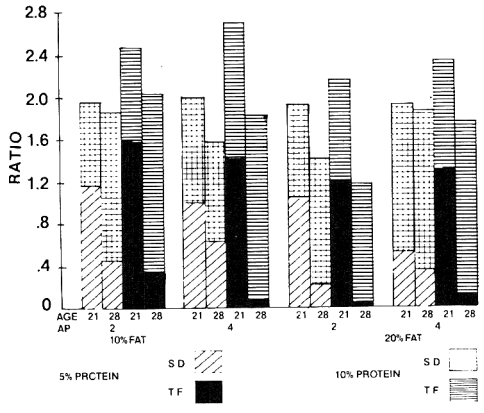

Figure 1. Effect of 7 experimental variables on 28-day unadjusted weight gain/protein consumption ratio for casein. Each bar contains data for both 5 and 10% protein levels. Values are means of replications 1 and 2. SD = ARS-Sprague-Dawley rats, TC = Taconic Farms rats, AP = acclimation period.

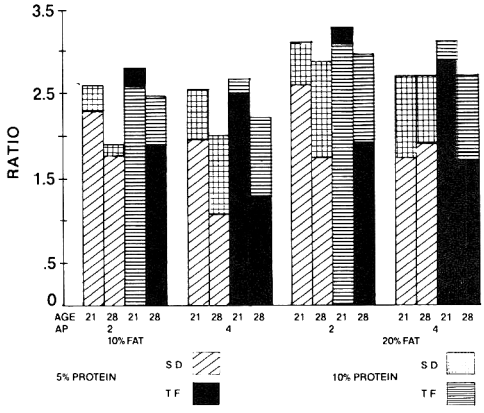

Figure 2. Effect of 7 experimental variables on 28-day unadjusted weight gain/protein consumption ratio for lactalbumin. Specifications: Figure 1.

the 6 g maximum spread between group means.

The 28-day weight gains for replicates 1 and 2 are summarized in Table 4. Although the average food consumption of most groups was higher in replicate 2 than in replicate 1, the weight gains were not consistently higher or lower in replicate 2.

Table 5 presents the mean unadjusted gain/consumption ratios for replicates 1 and 2. The mean ratios for 5% wheat flour were negative due to weight loss on this protein. The mean ratios of the 10% casein groups were much higher than the mean ratios of the 5% casein groups. The mean ratios for 5 and 10% protein from lactalbumin were similar, possibly indicating an excess of protein beyond the utilization capability of the animals.

Figures 1–3 show the 28-day mean unadjusted gain/consumption ratios for 5 and 10% casein (Figure 1), lactalbumin (Figure 2), and wheat flour (Figure 3). The values shown are the means of replicates 1 and 2. With casein (Figure 1), there was a large difference in mean ratio due to the protein level, as expected. The average values for the 28-day rats were significantly lower in every case than for the 21-day-old rats ($P < 0.001$). The mean ratios for the Taconic Farms rats were significantly higher ($P < 0.01$) than those for the ARS-Sprague-Dawley rats at the 10% protein level but were not different at the 5% protein level. The mean ratios at 20% fat were lower than at 10% fat at both protein levels ($P < 0.01$). Finally, the mean ratio after 4 days acclimation was not significantly different than after 2 days acclimation.

Figure 2 for lactalbumin shows a different type of response. At the 10% protein level, the mean ratio was only slightly higher than at 5% protein and in 2 cases (21 days-AP2-10% fat and 21 days-AP2-20% fat) was lower; the mean ratio for 28-day-old rats was less than for 21-day-old rats ($P < 0.001$); and the differences between 2 and 4 days acclimation were not significant at the 5% protein level but were significant at the 10% protein level ($P < 0.001$). Unlike the values with casein, the mean ratios with 10% fat were less than those with 20% fat at both the 5% protein level ($P < 0.05$) and the 10% protein level ($P < 0.001$). The ratios for ARS-Sprague-Dawley rats were significantly lower ($P < 0.05$) than for

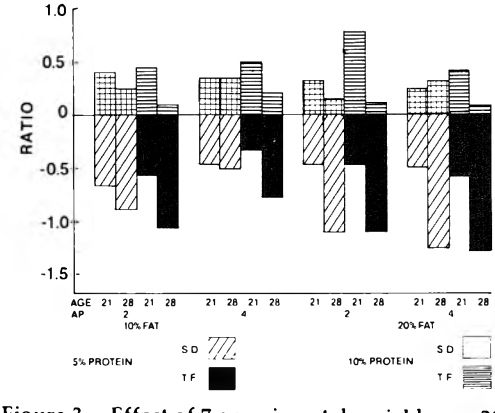

Figure 3. Effect of 7 experimental variables on 28-day unadjusted weight gain/protein consumption ratio for wheat flour. Specifications: Figure 1.

Table 6. Five-way analysis of variance of unadjusted weight gain/protein consumption ratios [a]

	Day 14						Day 21						Day 28					
	PL 5			PL 10			PL 5			PL 10			PL 5			PL 10		
Factor	C	L	W	C	L	W	C	L	W	C	L	W	C	L	W	C	L	W
Fat							*				*						*	
Acclim.	*			*												*		
Age			*	*				*				*				*		
Fat × acclim. × age				*														
RS																*		
Fat × RS										*								
Age × RS	*															*		
Acclim. × age × RS							*						*					*
Fat × acclim. × age × RS			*															
Rep.					*	*	*	*	*	*		*	*			*		*
Fat × rep.				*		*			*	*		*			*	*		
Acclim. × rep								*	*		*	*		*	*			*
Fat × acclim. × rep.					*	*			*						*			
Age × rep.	*						*			*			*					
Fat × age × rep.			*	*								*				*	*	
Acclim. × age × rep.											*	*				*		
Fat × acclim. × age × rep.						*						*	*					*
RS × rep.	*					*	*		*									*
Fat × RS × rep.						*			*						*			
Acclim. × RS × rep.						*												*
Fat × acclim. × RS × rep.						*			*									
Age × RS × rep.		*				*												
Fat × age × RS × rep.					*	*												
Acclim. × age × RS × rep.						*			*			*			*			
Fat × acclim. × RS × age × rep.					*	*					*	*					*	*

[a] * = Significant at $P < 0.05$. PL = protein level, C = casein, L = lactalbumin, W = wheat flour, acclim. = acclimation time, rep. = replication, RS = rat source.

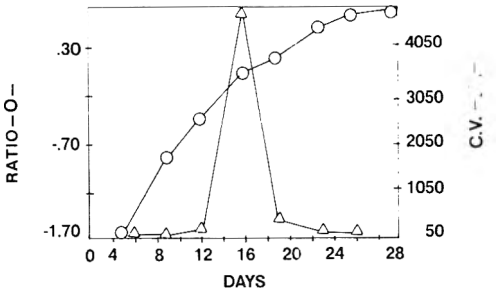

Figure 4. Mean unadjusted weight gain/protein consumption ratio (O) and CV (Δ) vs weigh days for 5% casein. Each point represents mean of 160 values.

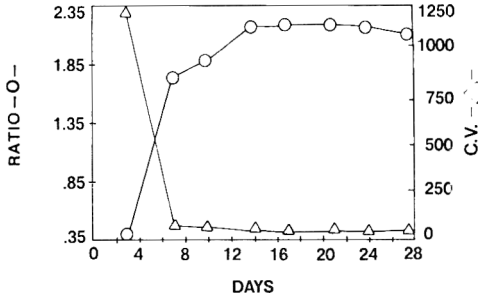

Figure 6. Mean unadjusted weight gain/protein consumption ratio (O) and CV (Δ) vs weigh days for 5% lactalbumin. Each point represents mean of 160 values.

Taconic Farms rats fed lactalbumin at both the 5 and 10% protein levels.

The mean ratios for wheat flour in Figure 3 are quite different from those for either casein or lactalbumin because wheat flour is a much poorer quality protein. At the 5% protein level, the mean ratios were below zero; at the 10% protein level, the mean ratios were still less than 1.0. As with casein, the mean ratios were significantly lower for 28-day-old rats ($P < 0.001$), ARS-Sprague-Dawley rats (at the 5% protein level only), and 20% fat (at the 5% protein level only). Acclimation period had only a slight, nonsignificant effect. In 2 cases, the mean ratios for 28-day rats were as high as or higher than those for 21-day-old rats (AP4-10% fat and AP4-20% fat). Although not shown in Figures 1-3, at 28 days there were significant differences ($P < 0.05$) between the mean ratios for the 2 replications in time for the 28-day ratios for 5% casein, 10% casein, and 10% wheat flour.

The results of the 5-way analysis of variance for the unadjusted gain/consumption ratios for 5 and 10% casein, lactalbumin, and wheat flour at 14, 21, and 28 days are given in Table 6. A large number of significant sources of variation are evident both from the 5 main factors (fat, acclimation period, rat source, rat age, and replication) and their interactions when tested within each protein source and protein level. At 28 days, the specified period for a PER assay, fat level was a significant source of variation for 10% lactalbumin; acclimation period, rat source, and age were significant for 10% casein; and replication was significant for 5% casein, 10% casein, and 10% wheat flour. A number of the possible interactions were also significant sources of variation. At the 10% protein level, the gain/consumption for casein—the control protein and level in the AOAC PER method—was subject to more variation (8 sources of significant variation) than wheat flour (6 sources of significant variation) or lactalbumin (4 sources).

Plots of mean gain/consumption ratios and CV of these ratios vs weigh days for the 3 proteins at 2 levels are shown in Figures 4-9. The data points for Figures 4-9 have been pooled for a particular protein and protein level. For the

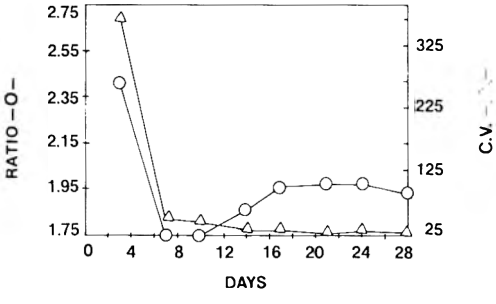

Figure 5. Mean unadjusted weight gain/protein consumption ratio (O) and CV (Δ) vs weigh days for 10% casein. Each point represents mean of 160 values.

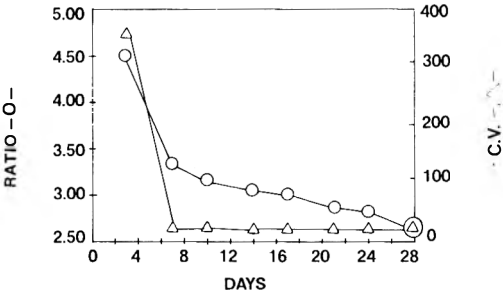

Figure 7. Mean unadjusted weight gain/protein consumption ratio (O) and CV (Δ) vs weigh days for 10% lactalbumin. Each point represents mean of 160 values.

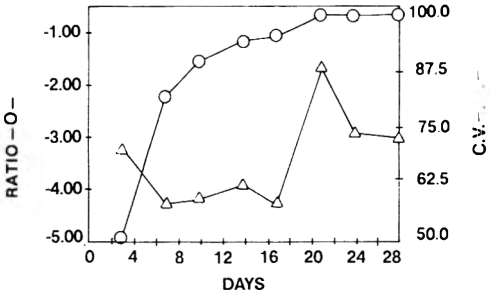

Figure 8. Mean unadjusted weight gain/protein consumption ratio (O) and CV (Δ) vs weigh days for 5% wheat flour. Each point represents mean of 160 values.

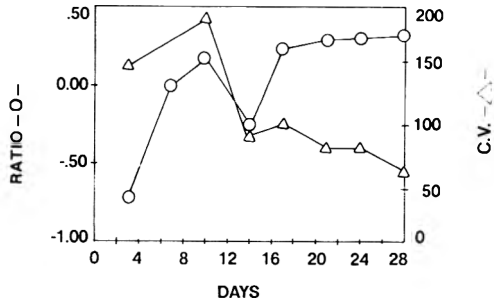

Figure 9. Mean unadjusted weight gain/protein consumption ratio (O) and CV (Δ) vs weigh days for 10% wheat flour. Each point represents mean of 160 values.

mean ratio, each point represents the mean of 160 values. The CV is an index of variability and is based on the pooled variation in measurements within the cells. Collectively, Figures 4-9 illustrate how the protein source and level affect the magnitude of average gain/consumption ratios and how the relative variability changes with time. In Figure 4, the mean ratio for 5% casein increases steadily with time throughout the 28-day period without leveling off. The CV takes an unexplained jump at 17 days but by the next weigh time returns to the previous value and reaches its lowest point at 28 days. The mean ratio for 10% casein (Figure 5) started high at 3 days, dropped to its lowest value at 7 days, leveled off at 17 days, and decreased after 24 days. The CV declined steadily with time and began to level off at 17 days.

With 5% lactalbumin (Figure 6), the mean ratio increased rapidly, reached a plateau at 14 days, and declined after 24 days. The CV started high at 3 days, dropped rapidly at 7 days, and reached a plateau at 17 days. The mean ratio for 10% lactalbumin (Figure 7) was at its highest value at 3 days and then began a steady decline all the way through 28 days. The CV reached its lowest point at 7 days and remained relatively constant through 28 days.

In Figure 8, the mean ratio for 5% wheat flour started at about −5.0 on day 3 and steadily increased until around day 21 but never reached 0. The CV for wheat flour was quite erratic over time and never decreased as with casein and lactalbumin. The mean ratio for 10% wheat flour (Figure 9) started below 0 at 3 days and increased to 0.25 at 28 days. The mean ratio still appeared to be increasing at 28 days, unlike the mean ratio of 10% casein or 10% lactalbumin, which was decreasing at 28 days. The CV decreased with time but never leveled off during the 28 days.

Because CV is a measure of variation, the optimum assay time could be defined as the time at which the CV has leveled off or reached its lowest point. If this criterion is applied, the optimum assay time for 10% casein, lactalbumin, and wheat flour would be 21, 7, and 28 days, respectively. For these proteins at the 5% level, the optimum times would be 28 days or more for casein and wheat flour and 14 or 17 days for lactalbumin. Thus, no one assay time is ideal for all proteins. However, 28 days does not seem to offer an increase in precision over 21 days. It would also appear from Figures 4-9 that the assay time in most cases should not be less than 21 days, if the protein level in the diet is 10%.

All of the results presented above have been based on ratios unadjusted for a system control. The gain/consumption ratios for lactalbumin and wheat flour (5 and 10% protein levels) at weigh days 14, 21, and 28 were adjusted according to the corresponding casein group (5 or 10%) set to a value of 2.5. The 5-way analyses of variance of these data are presented in Table 7. The only significant main effect was replication. The factors rat age, rat source, acclimation time, and fat level had no significant effect on the adjusted ratios at either 14, 21, or 28 days. This is in contrast to the analysis of variance of the unadjusted ratios (Table 7) in which all of these sources of variation were significant for at least one of the proteins at one or more weigh days. Thus, adjustment of a system control can eliminate these main sources of variance. However, a number of interactions of all 5 of the main effects were still significant. These observations suggest that there are other sources of experimental error that have not been identified and are not under uniform control between experiments.

The results of this study show that protein

Table 7. Five way analysis of variance of adjusted weight gain/protein consumption ratios [a]

	Day 14				Day 21				Day 28			
	PL 5		PL 10		PL 5		PL 10		PL 5		PL 10	
Factor	L	W	L	W	L	W	L	W	L	W	L	W
Fat × acclim. × age											*	
Fat × acclim. × RS						*						
Rep.							*				*	
Fat × rep.			*									
Age × rep.			*				*					
RS × rep.	*	*	*	*	*	*		*				*
Fat × RS × rep.	*	*		*	*	*		*			*	*
Acclim. × RS × rep.	*	*		*	*	*			*		*	
Fat × acclim. × RS × rep.	*	*	*	*	*		*		*	*	*	
RS × age × rep.	*	*	*	*	*	*		*		*		
Fat × RS × age × rep.	*	*		*	*	*					*	*
Acclim. × RS × age × rep.	*	*		*	*	*			*	*	*	
Fat × acclim. × RS × age × rep.	*	*		*	*	*	*	*	*	*	*	*

[a] Main effects and interactions that were significant for at least one set of the controlled factors. * = Significant at $P < 0.05$, PL = protein level, L = lactalbumin, W = wheat flour, acclim. = acclimation time, rep. = replication, RS = rat source.

source and protein level are the most significant factors influencing the gain/protein consumption ratio, evidence of the sensitivity of the test system. The next most important factor for the unadjusted ratio is the age of the rat. The use of rats varying in age from 21 to 28 days resulted in a significant source of variation ($P < 0.001$). This finding is in agreement with Chapman et al. (13). Not only is it more difficult to obtain tight weight ranges with 28-day-old rats, particularly after 4 days' adaptation, but the 28-day-old rats seem less responsive to differences in protein quality than the 21-day-old rats. On the basis of these differences, it is suggested that the AOAC procedure (6) be rewritten to specify the use of rats 21 days of age. The results of this study also suggest that the acclimation time should be fixed rather than a range. Based on this study, 2 days acclimation would be preferable to 4 days.

The replication factor was significant both with unadjusted and adjusted ratios and thus suggests that all conditions were not maintained uniformly in the study.

The plots of gain/consumption ratios vs days on test show that the ratios generally increase with time to a maximum and level off. The time at which the ratio reaches a maximum depends on both the source and level of the protein. It would appear from these plots that, in most cases, the time for the assay should not be less than 21 days if the protein level of the diet is 10%. Hackler (5) has presented data to show that adjusted PER values at 2 weeks are 97–103% of the values at 4 weeks, and thus the PER method could be shortened to 2 weeks without any loss of accuracy. However, the results of this study indicate that 2 weeks would be insufficient for a low-quality protein such as wheat flour.

The optimum assay time, as defined by the minimum CV, varied for the 3 proteins. The optimum assay time increased as the protein quality decreased. The normal 28-day assay time seemed to offer no significant improvement in precision over 21 days.

When the ratios for the test proteins are adjusted to a system control, casein, the effect of the single factors is cancelled out, but a number of interactions still are significant sources of variation.

Other means for evaluating protein quality from growth/consumption data, such as net protein ratio or slope ratio, have not been examined in this study and might provide different results from those presented here.[2]

Acknowledgments

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[2] Persons wishing to conduct further analysis of the data may obtain the tapes on loan from L.I.F.E., 915 15th St, NW, Washington, DC 20005.

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MICROBIOLOGICAL METHODS

Radiometric Microbiological Assay of Vitamin B_6: Assay Simplification and Sensitivity Study

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Modification of a previously developed radiometric microbiological assay for vitamin B_6 reduces assay complexity and time. Reduction of enzymatic treatment from 24 to 3 h essentially eliminates one day's time for the analysis of plasma samples. Use of lyophilized *Kloeckera brevis* cultures eliminates routine subculturing of the test organism, with no significant effect on test results. Modifications in test vial size and total volume in test vials have increased assay sensitivity to a level of 0.25 ng pyridoxine (PN), pyridoxal (PL), or pyridoxamine (PM) per vial level and decreased the amount of medium and labeled substrate (i.e., L-[1-^{14}C]-valine), thus reducing assay cost.

A radiometric microbiological assay (RMA) method for determining vitamin B_6 in blood and food has been reported (1, 2). The technique is based on measurement of $^{14}CO_2$ generated from the metabolism of L-[1-^{14}C]-valine by *Kloeckera brevis*. The amount of $^{14}CO_2$ produced is proportional to the amount of vitamin B_6 present in the test vial.

An inherent advantage of RMA over conventional turbidimetric microbiological assay is that it allows accurate measurement of $^{14}CO_2$ in the presence of precipitated food debris, plasma, or colored suspensions, thus allowing simplification of sample preparation. Measurement of $^{14}CO_2$ is automated at a rate of 1 test vial/min, using a gas flow system which contains an ionization chamber for quantitation of the radioactive carbon (Bactec 460, Johnston Laboratories, Cockeysville, MD).

A desirable characteristic of RMA is that it combines the biological specificity of the microorganism with the sensitivity and accuracy of radioactive decay measurement. It provides a simple and rapid tool for monitoring the response of a microorganism to a specific nutrient.

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One of the most commonly mentioned disadvantages of vitamin B_6 turbidimetric microbiological assay is that it is cumbersome and time-consuming. The already developed vitamin B_6 RMA is simpler and less time-consuming than the turbidimetric assay method (1). In this communication, results of studies to further simplify and increase sensitivity of vitamin B_6 RMA are presented.

Experimental

Apparatus and Reagents

(a) *Automatic freeze dryer.*—Virtis Co., Inc., Gardiner, NY.

(b) *Lyophilization ampules.*—Wheaton Scientific, Millville, NJ.

(c) *Spectrophotometer.*—Bausch and Lomb Spectronic 20.

(d) *Bactec 460.*—Johnston Laboratories, Cockeysville, MD.

Procedure

Lyophilization of Kloeckera brevis cells.—One or 2 loopsfull of rapidly growing *Kloeckera brevis* (ATCC 9774) cells were placed in 10 mL microinoculum broth (Difco Labs, Detroit, MI). Cells were dispersed by agitation and incubated 24 h at 30°C at a 5–10° angle from horizontal. After incubation, cells were centrifuged, washed once with sterile saline, and resuspended in 1 mL sterile saline.

One-tenth mL of this concentrated cell suspension was placed into sterile lyophilization ampules and pre-frozen in a dry ice (10% methanol) solvent bath. Cells were lyophilized at −40°C and 100 atm. Ampules were heat-sealed under the original vacuum while still connected to the drying chamber and then stored at 2–4°C until used.

Inoculum preparation.—Slant cultures of *K. brevis* in *Lactobacillus* Agar AOAC (Difco Labs) were incubated 24 h at 30°C. After incubation, the inoculum was prepared by aseptic transfer of *K. brevis* cells from the fresh agar slant to a color-

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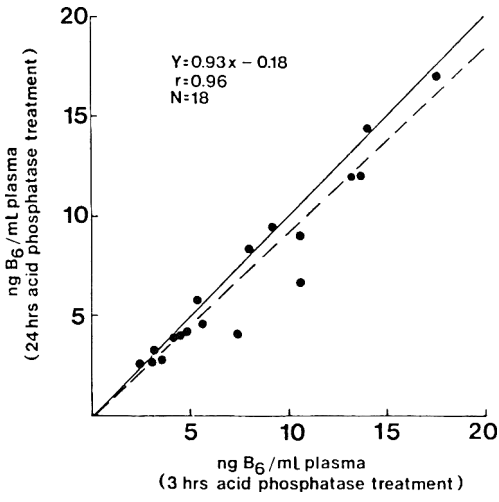

Figure 1. Comparison of 3 vs 24 h acid phosphatase treatment for dephosphorylation of plasma vitamin B_6 for radiometric microbiological assay method. Solid line represents identity. Dash line represents linear regression.

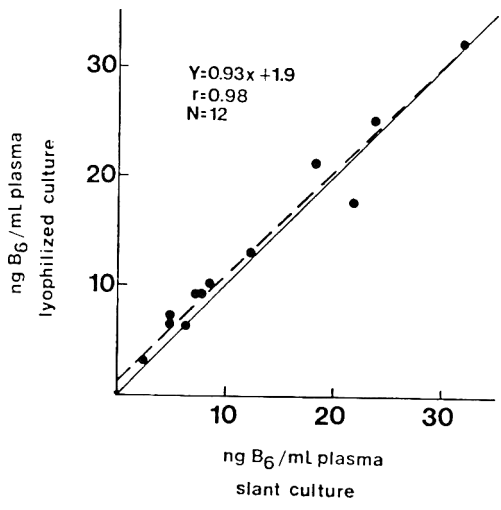

Figure 2. Comparison of lyophilized vs slant (normal) inoculum preparation of *Kloeckera brevis* cells for vitamin B_6 content using radiometric microbiological assay method. Solid line represents identity. Dash line represents linear regression.

imeter tube containing 5 mL of sterile 0.9% saline. Yeast cells were added to the tube until transmission was reduced by 25% at 640 nm (the spectrophotometer was set at 100% transmission with a saline blank). The cell suspension was transferred to an Erlenmeyer flask containing 95 mL sterile saline. One mL of this suspension was used as inoculum. For inoculum preparation with lyophilized *K. brevis* cells, contents of an ampule were reconstituted with 10 mL sterile saline, 0.1 mL was used as inoculum.

Dephosphorylation of plasma samples.—Dephosphorylation of plasma vitamin B_6 was performed as previously described (1), except that each plasma sample was treated with acid phosphatase for 3 and 24 h for comparison.

Sensitivity studies.—To increase assay sensitivity, a modification of the assay procedure was tested. To 20 mL serum vials (Arthur H. Thomas, Philadelphia, PA), 1 mL pyridoxine-Y-medium (Difco Labs), 0.25 and 0.5 mL of a 1 ng/mL PN, PL, or PM (equimolar) solution, and 0.1, 0.2, and 0.3 mL of a 10 ng/mL PN, PL, or PM (equimolar) solution was added. Total volume was made to 3 mL with deionized water. Vitamin B_6 concentration ranged from 0.25 to 3.0 ng/vial. Each vial was then injected with 0.25 μCi L-[1-^{14}C]-valine[1] (0.1 mL) and 0.1 mL lyophilized inoculum preparation and incubated at 30°C for 18–19 h. $^{14}CO_2$ produced was determined as previously described (1).

Radiometric microbiological assay of vitamin B_6.—Vitamin B_6 standard preparation, plasma collection, food sample preparation, and vitamin B_6 RMA procedure for plasma and food samples have been described elsewhere (1, 2).

Statistics.—Paired *t*-tests (3) were performed on data from Figures 1 and 2. Correlation coefficients (3) were obtained for data in Figures 1 and 2 and Table 1.

Results

Reduction of acid phosphatase enzymatic treatment time for dephosphorylation of plasma vitamin B_6.—Enzymatic treatment of plasma vitamin B_6 is usually allowed for 24 h. The desirability of having a shorter treatment time led to a comparison of enzymatic treatment of 3 vs 24 h. Figure 1 shows that no significant differences ($P > 0.2$) in vitamin B_6 values are obtained with either treatment.

Lyophilization of Kloeckera brevis cells.—The effect of using lyophilized cultures of *K. brevis* on RMA of vitamin B_6 for plasma or food was compared with the regular nonlyophilized (slant) inoculum preparation. Figure 2 and Table 1 indicate that no significant differences in assay results are obtained with either plasma or food samples ($P > 0.5$), using lyophilized *K. brevis* cultures in the analysis of vitamin B_6.

[1] Specific activity = 37.5 mCi/mmole. Research Product International, Elk Grove Village, IL.

Table 1. Comparison of vitamin B_6 values from various food samples, using lyophilized vs slant *Kloeckera brevis* cultures, with radiometric microbiological assay

Food	Vitamin B_6 content	
	Lyophilized	Slant
Cereals[a]		
Total	63.0	67.0
Fruity Pebble	32.0	36.5
Cornflakes	10.1	10.1
Most	70.5	76.5
Grape Nut Flakes	28.5	27.0
Bucwheats	31.0	34.5
Baby Food[b]		
Cereals & Egg	10.2	9.5
Chicken	50.5	53.5
Beets	11.6	10.1
Carrots	67.6	58.6
Spinach	34.3	32.9
Squash	46.4	43.6
Pork	75.2	80.0
Turkey	59.0	62.0
Baby Food—Juices[c]		
Orange	0.35	0.34
Apple-prune	0.23	0.24
Apple-cherry	0.39	0.42
Apple	0.16	0.18
Other Foods[a]		
Cottage cheese	0.13	0.15
Carnation milk	2.7	2.7

Overall correlation coefficient $r = 0.99$.
[a] μg PN equivalent/g.
[b] μg PN equivalent/jar.
[c] μg PN equivalent/mL.

Table 2. Radiometric microbiological standard curve of equimolar amounts of pyridoxine, pyridoxal, and pyridoxamine

Vitamin B_6 (ng)	Net metabolic index units[a] ($^{14}CO_2$ production)[b]		
	Pyridoxine	Pyridoxal	Pyridoxamine
0 (blank)	6	6	6
0.25	25	28	23
0.50	41	43	39
1.00	68	79	76
2.00	157	162	164
3.00	234	237	253

Lyophilized *Kloeckera brevis* and 0.25 μCi L-[1-^{14}C]-valine; incubation time 19 h at 30°C.
[a] 100 metabolic index units = 0.025 μCi.
[b] Average of duplicates.

Vitamin B_6 radiometric microbiological assay: Sensitivity studies.—Modifications in test vial size and total assay volume from the previously described RMA produced an increased assay sensitivity. We have been able to obtain a detection limit of 0.25 ng PN, PL, and PM/vial, and reduced the amount of L-[1-^{14}C]-valine from 0.5 to 0.25 μCi per vial (Table 2).

Discussion

Development of an RMA for total vitamin B_6, using *K. brevis*, has eliminated some of the problems and significantly reduced assay complexity encountered with presently available vitamin B_6 turbidimetric microbiological (yeast) assays (1, 2). Use of *K. brevis* as test organism for the determination of total vitamin B_6 eliminated the persistent problem of differential growth response to PN, PL, and PM encountered with *Saccharomyces uvarum* (4–6). Three different laboratories have independently shown that *K. brevis* responds essentially the same to equimolar concentrations of PN, PL, and PM (4, 5, 7).

The necessity of following growth (turbidity) with the turbidimetric microbiological assay method introduces limitations and complexity because colored, turbid, and precipitated materials must be removed to prevent interference with assay results. RMA eliminates this problem; these types of material do not interfere with the accurate measurement of $^{14}CO_2$ generated from yeast metabolism of labeled substrate. Instrumentation to measure $^{14}CO_2$ generated from catabolism of L-[1-^{14}C]-valine by *K. brevis* is automated and makes the analysis of a large number of samples a reality.

In these studies, we present further modifications of the vitamin B_6 RMA. For analysis of plasma samples, reducing enzymatic treatment from 24 to 3 h has essentially eliminated one day's time, with no statistically significant differences in test results ($P > 0.2$) (Figure 1). Use of lyophilized *K. brevis* cells has eliminated the routine subculturing of test organism with no significant differences ($P > 0.5$) in assay values when used for plasma or food vitamin B_6 analysis (Figure 2, Table 1). Lyophilized *K. brevis* cultures were stable for at least a year.

With minor modifications in test vial size (i.e., from 50 mL to 20 mL) and total volume in assay vials, sensitivity has increased to a detection level of 0.25 ng PN, PL, or PM/vial (see Table 2). The increased sensitivity is useful in the measurement of human plasma samples that fall in the low nanogram level. At the same time, it has reduced the amount of L-[1-^{14}C]-valine (from 0.5 μCi to 0.25 μCi), assay medium, and test vial size, thus reducing overall assay cost. Automated instrumentation (Bactec 460) for measuring

$^{14}CO_2$ is currently used in over 700 hospitals and clinical laboratories in the United States as well as industry (8).

The RMA method has significantly reduced assay complexity and time in the analysis of total vitamin B_6 in biological material. Further modifications presented in this communication have improved assay procedure, increased assay sensitivity, and reduced assay cost without compromising assay accuracy. RMA of vitamin B_6 can be a useful test in the clinical as well as the research laboratory.

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ALCOHOLIC BEVERAGES

Gas-Liquid Chromatographic Determination of Volatile Phenols in Matured Distilled Alcoholic Beverages

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Volatile phenols in matured spirits were determined by gas-liquid chromatography as their 2,4-dinitrophenyl derivatives. Phenol, o-cresol, *m*-cresol, *p*-cresol, guaiacol, *p*-ethylphenol, *p*-ethylguaiacol, eugenol, and *p*-(*n*-propyl)guaiacol, which occurs only in dark rum, were studied at concentrations ranging from 0 to 2 ppm depending on the phenol and the beverage. Scotch, Spanish, and Japanese whiskies, unlike other whiskies examined, contained *o*-, *m*-, and *p*-cresols. Cresols occur in whiskies as a consequence of the raw materials used, in particular peated malt. The level of *p*-ethylguaiacol is usually somewhat higher in cognac and other brandies than in whiskies. Dark rums differ from the other spirits examined in containing *p*-(*n*-propyl)guaiacol, and, typically, higher levels of *p*-ethylphenol, *p*-ethylguaiacol, and eugenol. These phenols also occur in higher quantities in some white rums than in whiskies and brandies. Two discriminant functions were obtained by discriminant analysis, which did not, however, allow the spirits to be classified adequately into their respective groups. By using these functions, 82% of the whiskies, 57% of the brandies, and 50% of the rums were correctly grouped.

Phenols are aroma components of foodstuffs whose characteristic presence is clearly discernible, especially in smoked and roasted products (1, 2). They are also present in various alcoholic beverages. Drawert et al. (3) have shown that beer contains several volatile phenolic compounds, and volatile phenols have also been reported in sherries and other wines (4–6). Matured distilled beverages also can contain phenols. Nishimura and Masuda (7) in Japan found a number of alkyl-substituted phenols among the acidic compounds from whiskies, while largely the same phenols have been isolated by both Dubois and Rigaud (8) and Timmer and co-workers (9) from the aroma components of rum.

Phenols produce a smoky aroma in alcoholic drinks and other foodstuffs, and at least part of the smoky character of Scotch whisky derives from phenolic compounds (10), although Bathgate and Taylor (11) reported that other components make a contribution as well.

Earlier work has concentrated mainly on the qualitative analysis of phenols in alcoholic beverages. There have been few reports of quantitative aspects, although Swan et al. (12) determined some volatile phenols in Scotch malt whiskies. The purpose of this work was to apply our previously developed method for determining phenolic compounds (13) to the analysis of matured distilled beverages. Discriminant and classification analysis was used to judge whether it would be possible to classify, even roughly, different beverages according to their phenol contents.

Experimental

Samples

Table 1 lists the 73 samples of commercial matured spirits that were investigated.

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard 5730A fitted with a ^{63}Ni electron capture detector and Hewlett-Packard 18740B inlet splitter. Operating conditions: temperatures—injection port 250°C, detector 300°C, oven held 4 min at 60°C, then programmed at 31°/min to 220°C and maintained at 220°C to end of run; gas flow rates—carrier gas (He) 1 mL/min, make-up gas (argon–methane, 95 + 5) 45 mL/min.

(b) *GLC column.*—Fused silica capillary (Hewlett-Packard), 50 m × 0.3 mm id, coated with SP-2100.

(c) *Integrator.*—Peak areas and quantitative results were calculated by Hewlett-Packard 3352B laboratory data system.

Reagents

(a) *1-Fluoro-2,4-dinitrobenzene.*—Fluka, Buchs, Switzerland.

(b) *n-Hexane.*—J.T. Baker, Deventer, The Netherlands.

(c) *2,2,4-Trimethylpentane.*—BDH Chemicals Ltd, Poole, UK.

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Table 1. Number and origin of matured spirits studied

Beverage	Origin	No. of brands
Whisky	Scotland	15
	Ireland	2
	United States	4
	Canada	1
	Japan	5
	Spain	1
	Argentina	2
	New Zealand	2
	India	1 (total 33)
Dark rum	Martinique	2
	Caribbean	1
	Guadeloupe	1
	Haiti	2
	Jamaica	1
Mixture of Finnish sulfite spirit and Martinique rum		1 (total 8)
White rum	Martinique	3
	Caribbean	1
	Guadeloupe	1
	Puerto Rico	2
	Cuba	1
	Demerara	1
	Brazil	1 (total 10)
Cognac	France	10
Mixture of Finnish sulfite spirit and cognac		1 (total 11)
Armagnac	France	1 (total 1)
Other brandies	France	2
	South Africa	2
	Italy	1
	Bulgaria	1
	Spain	1
	Soviet Union	1
	Greece	1
Mixture of brandy distillates from different countries		1 (total 10)
Grand total		73

(**d**) *Buffer.*—pH 11.5. Prepared according to Teorell and Stenhagen (14).

(**e**) *Standard solution of phenols.*—0.13 mg phenol, 0.08 mg *o*-cresol, 0.16 mg *m*-cresol, 0.06 mg *p*-cresol, 0.13 mg guaiacol, 0.10 mg *p*-ethylphenol, 0.13 mg 3,4-dimethylphenol (internal standard), 0.26 mg *p*-ethylguaiacol, 0.22 mg eugenol, and 0.22 mg *p*-(*n*-propyl)guaiacol in 1000 mL 45% aqueous ethanol.

(**f**) *Internal standard solutions.*—Stock solution: 0.43 mg 3,4-dimethylphenol/mL 45% aqueous ethanol. Working solution: 2.15 μg 3,4-dimethylphenol/mL 45% aqueous ethanol.

Calibration

Standard solution of phenols (20 mL), 1-fluoro-2,4-dinitrobenzene (1 mL of 2% ethanolic solution), and 10 mL pH 11.5 buffer were allowed to react in a pear-shape flask as described earlier (13). The 2,4-dinitrophenyl ethers of the phenols so produced were extracted with *n*-hexane. The hexane layer was separated, dried over Na_2SO_4, and 1 μL samples were taken for gas-liquid chromatographic (GLC) analysis. Response factors were calculated from peak areas for each of the phenols studied.

$$R = (A_{st}/A_{ph}) \times (c_{ph}/c_{st}) \quad (1)$$

where R = response factor; A_{st} = area of internal standard peak; A_{ph} = area of phenol derivative peak; c_{st} = amount of internal standard; c_{ph} = amount of phenol.

Determination

The 2,4-dinitrophenyl ether derivatives of the phenols in the beverages investigated were formed and analyzed as described previously (13), using 3,4-dimethylphenol as internal standard. Phenol contents were calculated as follows:

$$c_x = R \times (A_x/A_{st}) \times c_{st} \quad (2)$$

where R = response factor; A_x = area of phenol derivative peak; A_{st} = area of internal standard peak; c_x = amount of phenol studied (ppm); c_{st} = amount of internal standard (ppm).

Statistical Analysis

The results were examined by discriminant and classification analysis (15). Discriminant analysis produces functions that can best describe differences in the phenol contents of the beverages, and can reduce the original number of dimensions without losing information. Moreover, the derived linear functions may better satisfy a normal distribution than the original data. The proximity of data points to the calculated centroid of the groups was described by Mahalanobis' distance, such that values were classified into groups to make the distance minimum. The success of the classification was judged by posterior probability, which was maximum at the best division into groups. Statistical significance of differences between centroids of the groups was examined by the *F*-test.

Results and Discussion

Gas Chromatography

Gas chromatograms of the phenol derivatives show that whisky (Figure 1A) and dark rum (Figure 1B) contain largely the same volatile phenols. The only exception is *p*-(*n*-propyl)-guaiacol, which appears only in dark rum. Timmer et al. (9) also reported this guaiacol in

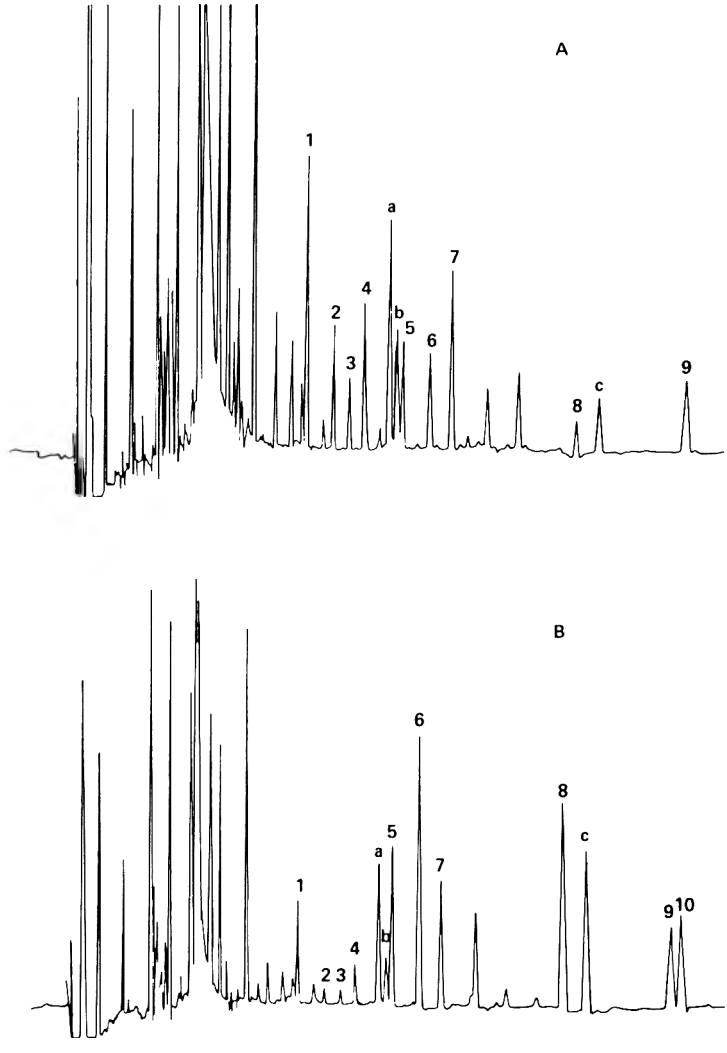

Figure 1. Phenols in whisky (A) and dark rum (B) chromatographed as 2,4-dinitrophenyl ethers: 1, phenol; 2, o-cresol; 3, *m*-cresol; 4, *p*-cresol; 5, guaiacol; 6, *p*-ethylphenol; 7, 3,4-dimethylphenol (internal standard); 8, *p*-ethylguaiacol; 9, eugenol; 10, *p*-(*n*-propyl)guaiacol. Peaks a, b, and c are unidentified compounds formed in the reaction.

rum. Cognac and other brandies (Figures 2A and 2B) contain the same phenols as whisky.

The formation mechanism and source of *p*-(*n*-propyl)guaiacol is not known, but it seems probable that maturing plays no part because it is absent from other matured spirits. It is possible that the bacteria used in the production of rum is involved in the appearance of *p*-(*n*-propyl)guaiacol in rum (16).

Quantitative Results

A comparison of the phenol composition of Scotch (Table 2) and other whiskies (Table 3) shows that cresols occur only in Scotch, Spanish, and Japanese whiskies. Raw materials are probably the source of cresols in Scotch whisky, because Scotch malt whisky is made only from peated malt (17), which contains cresols as major phenolic compounds (18, 19). When germinated barley is dried in peat smoke, the phenols formed by the pyrolysis of lignin are adsorbed on the surface of the grain (20) and are so carried through to the final product. The presence of cresols in Japanese and Spanish whiskies implies that peated malt is used in their production also. Similarly, the absence of cresols from the other

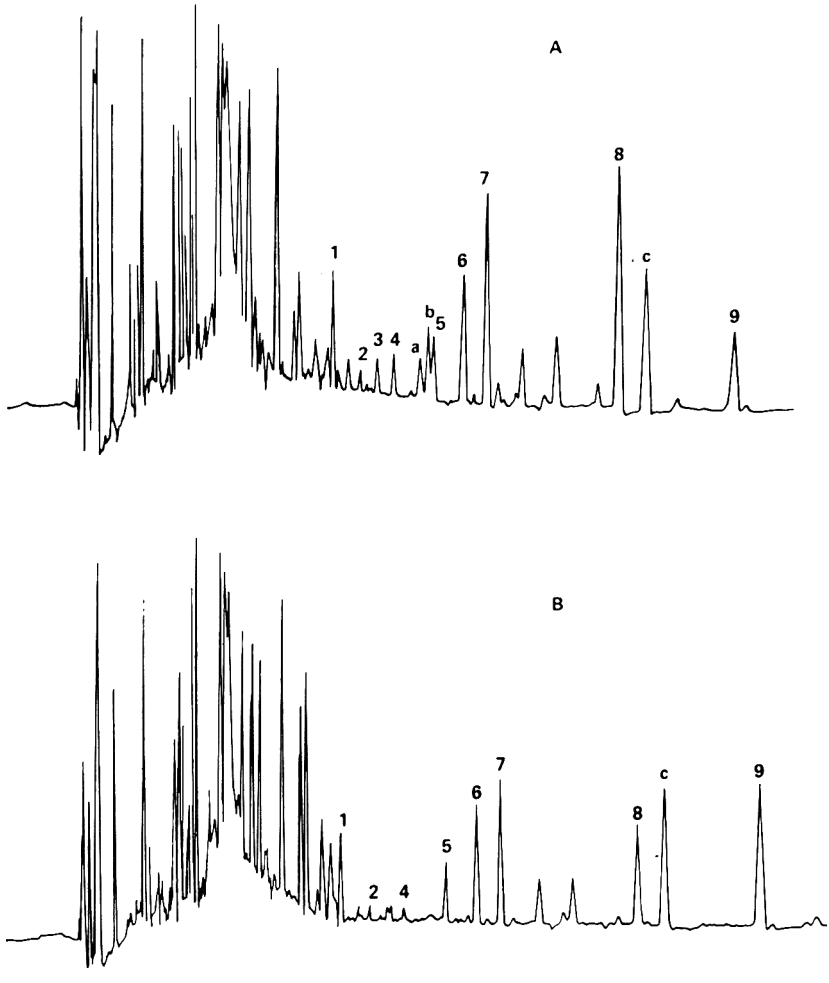

Figure 2. Phenols in cognac (A) and brandy (B) chromatographed as 2,4-dinitrophenyl ethers. See Figure 1 for peak identification.

Table 2. Concentration (ppm) of some volatile phenols in different commercial brands of Scotch whisky

Brand	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Guaiacol	*p*-Ethylphenol	*p*-Ethylguaiacol	Eugenol
1	0.12	0.07	0.03	0.06	0.07	0.04	0.03	0.03
2	0.18	0.15	0.05	0.08	0.12	0.08	0.05	0.12
3	0.17	0.13	0.05	0.08	0.11	0.06	0.06	0.18
4	0.14	0.11	0.04	0.06	0.09	0.05	0.04	0.12
5	0.05	0.03	0.01	0.02	0.03	0.04	0.06	0.10
6	0.25	0.20	0.08	0.11	0.13	0.06	0.05	0.04
7	0.05	0.04	0.01	0.02	0.03	0.04	0.02	0.04
8	0.02	0.02	tr[a]	tr	0.03	0.02	0.03	0.08
9	0.04	0.04	0.01	0.02	0.04	0.04	0.06	0.04
10	0.13	0.12	0.04	0.07	0.10	0.05	0.05	0.09
11	0.17	0.13	0.04	0.07	0.10	0.06	0.04	0.07
12	0.20	0.16	0.06	0.09	0.12	0.07	0.05	0.05
13	0.08	0.07	0.03	0.03	0.06	0.05	0.03	0.09
14	0.12	0.10	0.04	0.06	0.09	0.05	0.04	0.06
15	0.03	tr	tr	0.01	0.04	0.07	0.04	0.11

[a] tr = trace, 0.001 ppm < tr < 0.01 ppm.

Table 3. Concentration (ppm) of some volatile phenols in different commercial brands of whiskies originating outside Scotland

Origin	Brand No.	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Guaiacol	*p*-Ethylphenol	*p*-Ethylguaiacol	Eugenol
Ireland	1	0.01	ND[a]	ND	ND	0.02	0.01	0.01	0.06
	2	ND	ND	ND	ND	tr[b]	ND	ND	0.03
United States	1	0.02	tr	tr	tr	0.07	0.30	0.09	0.34
	2	tr	tr	tr	tr	0.06	tr	0.02	0.14
	3	0.02	ND	tr	tr	0.06	0.06	0.16	0.35
	4	tr	ND	ND	tr	0.02	0.06	0.06	0.05
Canada	1	tr	tr	tr	tr	0.01	0.05	0.03	0.05
Japan	1	0.04	0.02	0.01	0.01	0.03	0.03	0.02	0.03
	2	0.10	0.06	0.02	0.04	0.05	0.03	0.03	0.04
	3	0.08	0.04	0.02	0.03	0.03	0.02	tr	tr
	4	0.09	0.06	0.03	0.04	0.04	0.03	tr	tr
	5	0.05	0.05	0.03	0.03	0.04	0.06	0.05	0.06
Spain	1	0.09	0.07	0.03	0.04	0.06	0.03	0.05	0.04
Argentina	1	ND	ND	tr	tr	0.01	0.03	0.03	tr
	2	0.02	0.01	tr	tr	0.02	0.04	0.03	0.02
New Zealand	1	0.02	tr	tr	tr	0.09	0.05	0.15	0.58
	2	0.02	tr	tr	tr	0.09	0.05	0.16	0.56
India	1	0.15	tr	tr	0.01	0.05	0.01	0.02	0.08

[a] ND = none detected, < 0.001 ppm.
[b] tr = trace, 0.001 ppm < tr <0.01 ppm.

whiskies indicates that raw materials used in their production are not peated (17). The results reported here for Scotch whiskies agree well with those reported by Swan et al. (12) for Scotch malt whisky.

U.S. Bourbon whiskies differ from Scotch whiskies mainly in their higher content of eugenol (Table 3). New Zealand whisky resembles Bourbon in this respect. The type of oak barrel used for aging, and its previous history, can influence the eugenol content of the matured product. Thus, the amount of the compounds that can be extracted from the oak by the alcoholic mixture depends in large part on whether the barrel is new or on how many times it has been used. Occurrence of eugenol is typical of beverages matured in oak. Eugenol has been shown to be a main component of the phenolic fraction of an ethanolic extract of oak (21).

As would be expected, the phenol content of cognac and armagnac (Table 4) resembles that of the other brandies (Table 5). Moreover, they quite closely resemble the whiskies. The main differences are lower levels of cresols and far higher levels of *p*-ethylguaiacol.

The phenolic composition of rum (Table 6) is clearly different than that of the other spirits examined. The dark rums contain more alkyl-substituted phenols and guaiacols, and this is also true in many cases for the light-flavored white

Table 4. Concentration (ppm) of some volatile phenols in different commercial brands of cognac and armagnac

Beverage	Brand No.	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Guaiacol	*p*-Ethylphenol	*p*-Ethylguaiacol	Eugenol
Cognac	1	0.03	tr[a]	tr	tr	0.03	0.03	0.31	0.15
	2	0.04	tr	tr	tr	0.02	0.04	0.29	0.08
	3	0.03	tr	tr	tr	0.02	0.03	0.27	0.04
	4	0.04	tr	tr	tr	0.02	0.03	0.28	0.06
	5	0.01	tr	tr	tr	0.02	0.05	0.29	0.03
	6	0.05	ND[b]	tr	ND	ND	0.04	0.66	tr
	7	0.02	tr	tr	tr	0.01	0.03	0.29	0.07
	8	0.02	tr	tr	tr	tr	0.03	0.26	0.03
	9	0.05	0.02	0.02	tr	0.04	0.06	0.26	0.11
	10	0.03	tr	tr	tr	tr	0.04	0.32	0.03
Mixture of Finnish sulfite spirit and cognac		0.02	tr	tr	tr	tr	tr	0.20	tr
Armagnac	1	0.05	tr	tr	tr	tr	0.09	0.24	0.12

[a] tr = trace, 0.001 ppm < tr < 0.01 ppm.
[b] ND = none detected, <0.001 ppm.

rums. No reason for this difference has been confirmed but as noted earlier it is probably connected with the bacteria used in the production of rum (16).

Although eugenol and *p*-(*n*-propyl)guaiacol derive from different sources, they occur at similar concentrations in dark rums and resemble each other in structure. In eugenol, the *p*-substituent is an unsaturated alkyl group; the guaiacol contains the saturated analog, *n*-propyl.

Recovery of Phenols and Repeatability of Method

The suitability of the method for determining phenols in alcoholic beverages was tested by measuring the phenols present and then adding known amounts of each phenol and carrying out another determination. Results in Table 7 show that recoveries were excellent. The repeatability of the method was also good; the coefficient of variation based on 4 independent determinations was 6%.

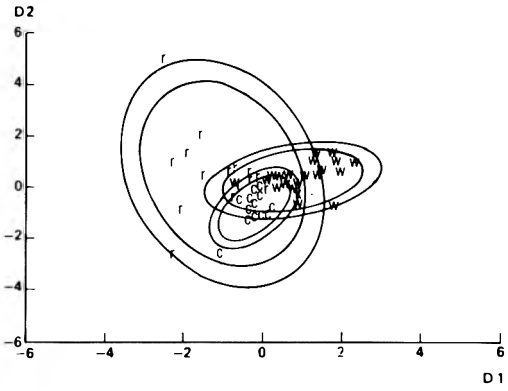

Figure 3. Classification by discriminant analysis on the basis of concentration of phenols in whisky (w), rum (r), and cognac (including other brandies) (c). Inner ellipses contain 95% of the samples and outer contain 99%. Coordinates of the centroids are: whisky (0.724, 0.241), rum (−1.058, 0.512), and brandy (−0.231, −0.819). Total discriminating power of variables, $F_{16,124}$ = 5.89, is significant at the 1% level.

Discriminant Analysis

Discriminant analysis was applied to the results to examine whether phenol content could be used to assign a beverage to its appropriate class. Two-variable discriminant functions resulted, as illustrated in Figure 3. The corresponding average values of the discriminant functions were significantly different by the *F*-test at the 1% level. Cognac was included with the other brandies for these analyses.

The contents of *o*-cresol, *p*-ethylphenol, and *p*-ethylguaiacol proved to be the most discriminating variables for distinguishing the spirits studied. *p*-Ethylguaiacol, however, was less useful than the other two. Whiskies and rums can be classified with reasonable ease; the brandies seem to occupy the middle group and overlap with the other classes far more (Figure 3).

The most significant difference between rums and whiskies is the markedly higher levels of *p*-ethylphenol and *p*-ethylguaiacol in the rums, which also contain, on the average, somewhat more guaiacol. The cresol content of rums is

Table 5. Concentration (ppm) of some volatile phenols in different commercial brands of brandy other than cognac and armagnac

Origin	Brand No.	Phenol	o-Cresol	*m*-Cresol	*p*-Cresol	Guaiacol	*p*-Ethylphenol	*p*-Ethylguaiacol	Eugenol
France	1	0.04	tr[a]	tr	tr	0.02	0.15	0.17	0.04
	2	0.04	tr	tr	tr	tr	0.07	0.09	tr
South Africa	1	0.04	ND[b]	0.02	ND	0.02	0.01	0.03	0.05
	2	0.03	tr	tr	tr	tr	0.02	0.01	0.05
Italy	1	0.04	tr	tr	tr	tr	0.14	0.17	0.05
Bulgaria	1	0.07	0.02	0.01	tr	0.06	0.08	0.11	0.27
Spain	1	0.03	tr	tr	tr	0.01	0.08	0.10	0.05
Soviet Union	1	0.06	tr	tr	tr	0.02	0.16	0.19	0.07
Greece	1	0.01	tr	tr	tr	0.02	0.03	0.15	tr
Mixture of brandy distillates from different countries		0.04	ND	ND	ND	tr	0.09	0.20	tr

[a] tr = trace, 0.001 ppm < tr < 0.01 ppm.
[b] ND = none detected, <0.001 ppm.

Table 6. Concentration (ppm) of some volatile phenols in different commercial brands of dark and white rums

Origin	Brand No.	Phenol	o-Cresol	*m*-Cresol	*p*-Cresol	Guaiacol	*p*-Ethylphenol	*p*-Ethylguaiacol	Eugenol	*p*-(*n*-Propyl)guaiacol
Dark Rum										
Martinique	1	0.19	0.07	0.04	0.09	0.80	1.89	1.13	0.89	0.69
	2	0.19	0.05	0.03	0.20	0.83	1.96	1.32	1.36	0.84
Caribbean	1	0.12	tr[a]	tr	0.11	0.56	1.64	1.15	1.00	0.61
Haiti	1	0.11	0.02	0.02	0.03	0.10	0.05	0.09	0.02	tr
	2	0.16	tr	tr	0.03	0.10	0.09	0.10	tr	tr
Guadeloupe	1	0.26	0.10	0.05	0.08	0.78	1.33	1.83	0.97	0.64
Jamaica	1	0.03	tr	tr	tr	0.11	0.07	0.13	0.05	0.07
Mixture of Finnish sulfite spirit and Martinique rum		0.03	tr	tr	tr	0.09	0.21	0.14	0.12	0.10
White Rum										
Martinique	1	0.04	ND[b]	tr	tr	0.13	0.88	0.88	0.10	ND
	2	0.06	0.02	0.01	0.02	0.13	0.50	0.33	0.11	ND
	3	0.11	tr	0.02	0.02	0.13	0.56	1.04	0.06	ND
Caribbean	1	0.01	ND	tr	tr	0.01	0.12	0.02	tr	ND
Guadeloupe	1	0.03	tr	tr	tr	0.12	0.21	0.48	0.18	ND
Puerto Rico	1	0.01	ND	ND	ND	0.03	0.01	0.03	tr	ND
	2	tr	ND	ND	ND	tr	0.05	tr	tr	ND
Cuba	1	0.02	ND	ND	ND	0.02	0.02	tr	ND	ND
Demerara	1	tr	ND	ND	ND	ND	ND	ND	ND	ND
Brazil	1	0.02	ND	ND	ND	ND	ND	ND	ND	ND

[a] tr = trace, 0.001 ppm < tr < 0.01 ppm.
[b] ND = none detected, <0.001 ppm.

Table 7. Recovery of phenols (ppm) added to spirits

Phenol	Whisky		Rum		Cognac	
	Added	Recd	Added	Recd	Added	Recd
Phenol	0.13	0.12	0.13	0.12	0.13	0.12
o-Cresol	0.08	0.10	0.08	0.08	0.08	0.09
m-Cresol	0.16	0.17	0.16	0.16	0.16	0.14
p-Cresol	0.06	0.07	0.06	0.06	0.06	0.06
Guaiacol	0.13	0.13	0.13	0.14	0.13	0.12
p-Ethylphenol	0.10	0.11	0.10	0.11	0.10	0.10
p-Ethylguaiacol	0.26	0.29	0.26	0.27	0.26	0.26
Eugenol	0.22	0.24	0.22	0.23	0.22	0.22
p-(*n*-Propyl)guaiacol	—	—	0.22	0.22	—	—

much lower than that of whiskies, largely because of the contribution of Scotch whisky.

The phenol content of the brandies resembles that of the other beverages except for the cresols, which are generally lower in brandies, and *p*-ethylguaiacol, which is, on the average, clearly higher in rums and lower in whiskies than in brandies.

Assigning the beverages studied to their respective classes on the basis of the discriminant analysis of their phenol contents was generally successful (Table 8). In addition to clear assignments, there were several cases for which the probabilities of belonging to more than one class were similar. The whiskies were assigned most successfully, while the brandies especially were often incorrectly assigned because their phenol content closely resembled that of another group. Correct assignments were made for 82% of the whiskies, 50% of the rums, and 57% of the brandies.

Conclusion

The concentrations of volatile phenols are somewhat different in whiskies, rums, cognacs, and other brandies. The main difference between the rums and other beverages is the presence of *p*-(*n*-propyl)guaiacol, which occurs only in the dark rums. The contents of *o*-cresol, *p*-ethylphenol, and *p*-ethylguaiacol appear to be the most influential separators. The differences, however, are not sufficient to classify the alco-

Table 8. Success of classifying whiskies, rums, and brandies into their respective groups on the basis of their levels of volatile phenolic compounds

Beverage	No. of brands	No. of brands correctly classified by Mahalanobis' distance[a]	No. of uncertain classifications by Mahalanobis' distance[b]	No. of brands correctly classified by posterior probability[c]	No. of uncertain classifications by posterior probability[d]
Whisky					
Scotch	15	15	0	14	1
Irish	2	0	2	2	0
United States	4	3	1	3	1
Canadian	1	1	0	0	1
Japanese	5	5	0	4	1
Spanish	1	1	0	1	0
Argentina	2	0	2	1	1
New Zealand	2	2	0	2	0
India	1	0	0	0	1
Total	33	27 (82%)	5 (15%)	27 (82%)	6 (18%)
Dark rum	8	8	0	6	1
White rum	10	8	2	3	1
Total	18	16 (89%)	2 (11%)	9 (50%)	2 (11%)
Cognac	11	10	0	10	0
Brandy	10	2	4	6	2
Total	21	12 (57%)	4 (19%)	16 (76%)	2 (10%)

[a] Mahalanobis' distance is clearly the shortest.
[b] No clear difference in Mahalanobis' distances between the groups.
[c] Posterior probability is clearly the greatest.
[d] No clear difference in posterior probabilities between the groups.

holic beverages studied into their respective groups with adequate precision. Correct assignments by the discriminant analysis vary between 50 and 80% depending on the type of beverage. Raw materials have little effect on the phenol content of the product, especially for the different types of whisky.

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High Performance Liquid Chromatographic Determination of Nonvolatile Phenolic Compounds in Matured Distilled Alcoholic Beverages

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Gallic acid, vanillic acid, syringic acid, vanillin, syringaldehyde, and ferulic acid were determined in matured spirits by reverse phase high performance liquid chromatography. Concentrations varied from 0 to 11 ppm depending on the compound and the beverage. Gallic acid and syringaldehyde were the main components. Vanillic acid, vanillin, and, especially, gallic acid were the most important in distinguishing the different classes of spirit. Discriminate analysis gave 2 factors, which, however, could not be used to classify the samples with any great confidence: 61% of the whiskies, 33% of the rums, and 52% of the brandies were correctly assigned in this way.

A number of nonvolatile phenolic compounds are present in many food products. During maturing of spirits, phenolic compounds are formed by alcoholysis of lignin in the oak of the barrels. Lignin, which is soluble in alcohol, tends to dissolve in the beverage, and, when it decomposes, forms mainly phenolic compounds (1, 2). Nonvolatile phenolic acids and aldehydes have been reported in beers, wines, whiskies, and armagnac (3–6); in beer, they help to retard oxidation (7).

Although the process of maturing and the compounds thus formed are of exceptional importance for alcoholic beverages, the content of nonvolatile compounds in spirits has attracted little attention. Most previous determinations of nonvolatile phenolic compounds have been based on gas chromatography (8). Because these compounds are difficult to vaporize and many are readily decarboxylated at elevated temperatures, it has been necessary to form derivatives before analysis. This has been most commonly done by silylation, for example with bis(trimethylsilyl)acetamide (9). The troublesome derivatization, however, has been increasingly avoided by using high performance liquid chromatography, a technique that has the further advantage of being carried out at lower temperatures (10–13).

The purpose of this work was to apply liquid chromatography to the determination of nonvolatile acids and aldehydes, particularly in matured distilled beverages. Discriminant and classification analysis was applied to the results to discover whether it would be possible to classify the different beverages on the basis of their concentration of nonvolatile phenolic compounds.

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Experimental

Samples

Table 1 lists the 70 different commercial spirits that were examined.

Apparatus

(a) *Liquid chromatograph.*—Hewlett-Packard 1084B. Conditions: oven temperature 40°C; mobile phase flow rate 1 mL/min.

(b) *Detector.*—Variable wavelength (190–600 nm) UV-Vis, linear over the range used. Wavelengths used are shown in Table 2.

(c) *Injector.*—Variable volume, 0–200 μL.

(d) *HPLC column.*—25 cm × 0.4 cm id packed under pressure with Lichrosorb RP-18 particle size 7 μm, (E. Merck, Darmstadt, GFR).

Eluants

(a) *Eluant A.*—Water–acetic acid (980 + 20, v/v), containing 0.02M sodium acetate.

(b) *Eluant B.*—Water–methanol–1-propanol–acetic acid (815 + 140 + 25 + 20, v/v), containing 0.02M sodium acetate.

(c) *Gradient.*—4 min with mixture of 10% B in A, then proportion of B was increased steadily for 16 min to 100%, which was then used until the end of the run. The gradient profile is illustrated in Figure 1. Eluants were filtered through a Millipore membrane (0.8 μm), and degassed by heating under vacuum before use.

Reagents

(a) *Methanol.*—For liquid chromatography (Orion Corp. Ltd, Espoo, Finland).

(b) *1-Propanol.*—Pro analyse, E. Merck, Darmstadt, GFR.

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Table 1. Number and origin of matured spirits studied

Beverage	Origin	No. of brands
Whisky	Scotland	15
	Ireland	2
	United States	4
	Canada	1
	Japan	5
	Spain	1
	Argentina	2
	New Zealand	2
	India	1 (total 33)
Dark rum	Martinique	2
	Caribbean	1
	Guadeloupe	1
	Haiti	1
	Jamaica	1
Mixture of Finnish sulfite spirit and Martinique rum		1 (total 7)
White rum	Martinique	1
	Caribbean	1
	Guadeloupe	1
	Puerto Rico	2
	Cuba	1
	Demerara	1
	Brazil	1 (total 8)
Cognac	France	10
Mixture of Finnish sulfite spirit and cognac		1 (total 11)
Armagnac	France	1 (total 1)
Other brandies	France	2
	South Africa	2
	Italy	1
	Bulgaria	1
	Spain	1
	Soviet Union	1
	Greece	1
Mixture of brandy distillates from different countries		1 (total 10)
Grand total		70

(c) *Acetic acid.*—Pro analyse, E. Merck.

(d) *Sodium acetate trihydrate.*—Pro analyse, E. Merck.

(e) *Water.*—Distilled and deionized, filtered through Millipore membrane (0.45 μm).

(f) *Standard solution of phenolic compounds.*—5 mg gallic acid, protocatechuic acid, vanillyl alcohol, *p*-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzyl alcohol, vanillic acid, syringic acid, vanillin, syringaldehyde, ferulic acid, and ethyl vanillin (internal standard) in 1 L 45% aqueous ethanol.

(g) *Ethyl vanillin standard solutions.*—Stock solution: 1.25 mg/mL 45% aqueous ethanol; working solution: 0.125 mg/mL 45% aqueous ethanol.

Calibration

Evaporate 50 mL standard solution to 10 mL, filter solution through 0.8 μm Millipore membrane, and use 10 μL for injection volume. Response factors for each of the compounds investigated were calculated from peak areas and the known concentrations (14).

Determination

Add 2 mL ethyl vanillin standard solution (internal standard) to 50 mL sample beverage, evaporate solution to about 10 mL on rotary evaporator, and filter through Millipore filter (0.8 μm). Inject 10 μL filtrate for analysis.

Statistical Analysis

Discriminant and classification analysis was used to produce functions that best described differences between the spirits (15). Statistically significant differences between the centroids of the groups were judged by the *F*-test.

Results and Discussion

Liquid Chromatography

A chromatogram of the standard phenolic alcohols, acids, and aldehydes is shown in Figure 1. It can be seen that the elution gradient and column used produce good resolution down to

Table 2. Relative retention times (*RRT*) of phenols vs ethyl vanillin, absorption maxima of compounds studied, wavelengths used, and ratios of absorbances measured at 275 and 300 nm

Phenolic compound	*RRT*	Absorption max., nm	Wavelength used, nm	A_{275}/A_{300} ref.	A_{275}/A_{300} Whisky	Rum	Cognac
Gallic acid	0.138	270	270	2.7	2.4	2.4	2.4
Protocatechuic acid	0.260	258, 292	270	1.2	—	—	—
Vanillyl alcohol	0.385	275	270	—			
p-Hydroxybenzoic acid	0.436	254	270	—			
3-Hydroxy-4-methoxy-benzyl alcohol	0.514	275	270	—			
Vanillic acid	0.570	260, 290	260	1.4	1.3	1.6	1.6
Syringic acid	0.640	270	270	2.3	2.5	2.1	2..
Vanillin	0.713	280	280	1.1	1.2	1.2	1.3
Syringaldehyde	0.773	304	304	0.4	0.4	0.3	0.4
Ferulic acid	0.871	320	320	0.6	—	—	—
Ethyl vanillin	1.000	270, 312, 358	280	—	—	—	—

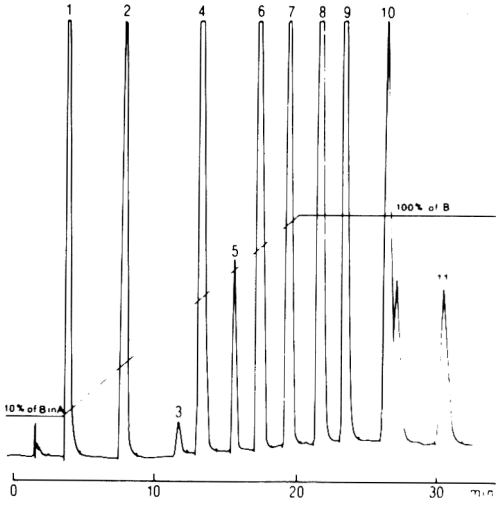

Figure 1. Liquid chromatogram of standard solution: 1, gallic acid; 2, protocatechuic acid; 3, vanillyl alcohol; 4, *p*-hydroxybenzoic acid; 5, 3-hydroxy-4-methoxybenzyl alcohol; 6, vanillic acid; 7, syringic acid; 8, vanillin; 9, syringaldehyde; 10, ferulic acid; 11, ethyl vanillin (internal standard). The gradient profile is shown in the chromatogram: 10% of B in A to 100% of B.

the baseline. It is also apparent that retention time is determined by polarity, with the most polar eluting first. Thus the most polar, gallic acid, elutes first, and the least polar, ferulic acid, last. The alcohols and aldehydes behave similarly, with the more polar vanillin eluting before syringaldehyde. The retention times of the compounds investigated (relative to ethyl vanillin) are given in Table 2.

The chromatograms in Figure 2 show that whisky, rum, and cognac contain largely the same compounds; chromatograms of the other brandies are similar. Rum and cognac contain some unidentified compounds.

Ultraviolet Spectroscopy

Absorption maxima in the UV spectra of the standard phenolic components are presented in Table 2. UV spectroscopy can be used to support other means of identifying components. Thus, the ratio of absorbance at 275 and 300 nm is given in Table 2 for authentic compounds and also for those components in the samples, identified by LC retention time. Agreement is good.

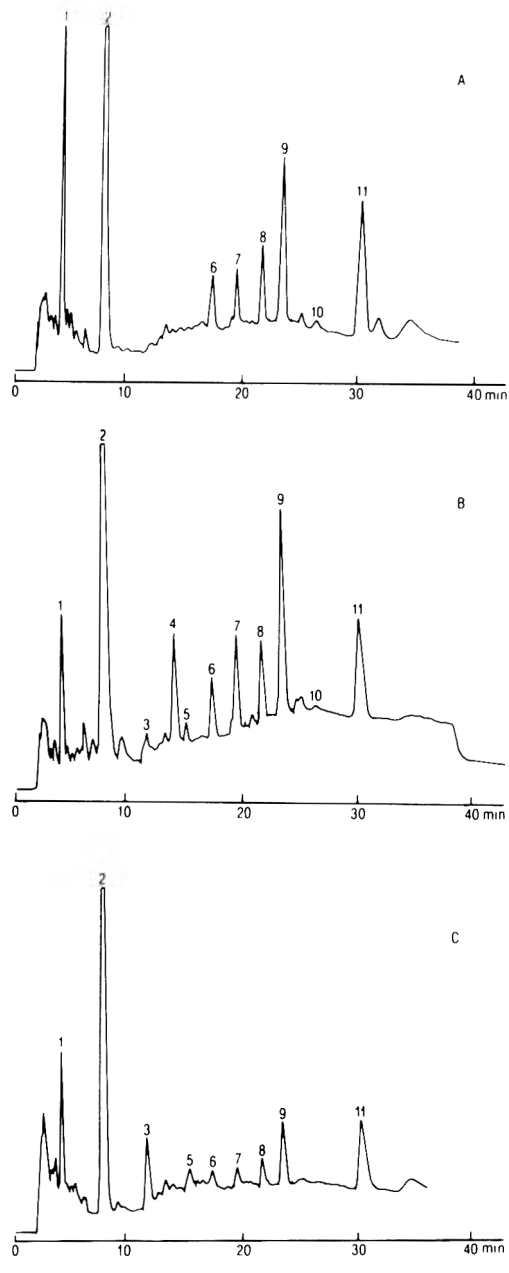

Figure 2. Liquid chromatogram of phenolic compounds in whisky (A), rum (B), and brandy (C): 1, gallic acid; 2, unknown; 3, unknown; 4, unknown; 5, unknown; 6, vanillic acid; 7, syringic acid; 8, vanillin; 9, syringaldehyde; 10, ferulic acid; 11, ethyl vanillin (internal standard).

Quantitative Results

There is little difference between the whiskies (Tables 3 and 4) in their content of nonvolatile compounds. The only real exception is the clearly higher level of syringaldehyde in the U.S. whiskies, which also contain slightly more vanillin. The results are in good agreement with

Table 3. Concentration (ppm) of some nonvolatile phenolic compounds in different commercial brands of Scotch whisky

Brand No.	Gallic acid	Vanillic acid	Syringic acid	Vanillin	Syringaldehyde	Ferulic acid
1	0.88	0.13	0.24	0.31	0.67	ND[a]
2	3.14	0.47	0.62	0.84	1.93	tr[b]
3	5.72	0.61	1.08	1.15	2.44	tr
4	4.75	0.85	1.24	1.23	2.67	tr
5	5.75	0.78	1.25	1.09	2.54	tr
6	1.97	0.33	0.68	0.67	1.30	tr
7	1.25	0.28	0.42	0.63	1.37	tr
8	2.87	0.31	0.47	0.72	1.46	tr
9	1.11	0.18	0.32	0.59	1.12	ND
10	3.16	0.39	0.56	0.80	1.73	tr
11	2.08	0.16	0.44	0.60	1.21	ND
12	2.56	0.23	0.31	0.41	0.97	tr
13	6.09	0.44	0.84	1.01	2.22	ND
14	2.11	0.40	0.72	0.85	1.64	ND
15	4.28	0.82	1.43	2.06	4.04	tr

[a] ND = none detected.
[b] tr = trace, <0.01 ppm.

those reported by Baldwin et al. (4). Gallic acid is the major compound in Scotch whisky (Table 3), with the contents of syringic acid and syringaldehyde also generally higher than that of other components.

The cognacs and armagnac (Table 5) and other brandies (Table 6) broadly resemble the whiskies, although the concentration of gallic acid is generally higher. The results agree with those of Puech et al. (6).

The levels of nonvolatile phenolic compounds in dark rums are similar to those in the other spirits, with products from Haiti and Guadeloupe containing somewhat more vanillin, syringic acid, and syringaldehyde than the other dark rums (Table 7). In contrast, the white rums contain markedly fewer phenolic compounds than do the other samples; vanillin, syringaldehyde, and syringic acid are found only in some of the products. The difference between the dark and white rums is probably a result of different production methods.

The length of the maturing period has some effect on the nonvolatile phenolic compounds present in a beverage; products that stay longer in oak barrels generally contain more. This,

Table 4. Concentration (ppm) of some nonvolatile phenolic compounds in different commercial brands of whiskies originating outside Scotland

Origin	Brand No.	Gallic acid	Vanillic acid	Syringic acid	Vanillin	Syringaldehyde	Ferulic acid
Ireland	1	3.38	0.41	0.65	0.66	1.55	tr[a]
	2	1.40	0.26	0.35	0.47	1.07	ND[b]
United States	1	6.81	1.07	2.21	2.19	6.59	0.10
	2	2.41	1.87	3.57	3.35	10.21	tr
	3	6.81	1.34	2.61	1.95	6.91	tr
	4	ND	0.73	1.18	2.07	4.08	ND
Canada	1	2.93	0.62	1.08	0.98	2.44	tr
Japan	1	ND	0.41	0.18	0.60	1.51	ND
	2	0.26	0.15	0.25	0.39	0.79	ND
	3	ND	0.19	0.18	0.26	0.51	ND
	4	ND	tr	tr	0.04	0.28	ND
	5	0.26	0.44	0.68	0.78	1.79	tr
Spain	1	1.05	0.07	0.14	0.26	0.61	ND
Argentina	1	ND	0.77	1.23	1.17	2.65	ND
	2	ND	0.29	0.47	0.62	1.40	ND
New Zealand	1	1.71	0.58	1.04	2.38	3.27	tr
	2	1.74	0.61	1.08	2.40	3.31	tr
India	1	ND	0.04	ND	5.96	ND	ND

[a] tr = trace, <0.01 ppm.
[b] ND = none detected.

Table 5. Concentration (ppm) of some nonvolatile phenolic compounds in different commercial brands of cognacs and armagnac

Beverage	Brand No.	Gallic acid	Vanillic acid	Syringic acid	Vanillin	Syringaldehyde	Ferulic acid
Cognac	1	4.20	0.22	0.57	0.81	2.04	tr[a]
	2	10.82	0.46	1.09	1.21	3.75	ND[b]
	3	7.70	0.27	0.55	0.82	2.12	ND
	4	6.32	0.43	0.82	1.11	2.91	ND
	5	2.92	tr	0.20	0.45	0.74	ND
	6	3.80	0.26	0.61	0.42	1.30	ND
	7	6.69	0.25	0.53	0.75	1.81	ND
	8	2.54	0.17	tr	0.50	0.69	ND
	9	9.52	0.48	0.88	1.85	3.19	ND
	10	2.28	tr	tr	0.34	0.41	ND
Mixture of Finnish sulfite spirit and cognac	1	2.32	tr	0.12	0.24	0.60	ND
Armagnac	1	3.42	0.12	0.19	0.25	0.67	ND

[a] tr = trace, <0.01 ppm.
[b] ND = none detected.

however, is complicated because the ease of extraction of the phenols is affected by the type of oak and the way in which the inner surface of the barrel has been treated.

Repeatability of the Method

Six independent determinations of the compounds in the standard mixture gave an average standard error of the mean of 2%. The suitability of the method for determining nonvolatile phenolic compounds in alcoholic beverages was confirmed by adding known amounts of standard solution to a whisky and measuring the recoveries (Table 8).

Discriminant Analysis

Discriminant analysis was used to find the best factors to describe differences between the beverages. The results were 2-variable functions, which are plotted in Figure 3. The difference between the functions is significant at the 1% level (F-test). In this analysis, all brandies were grouped together. The main distinguishing features between beverages were the levels of gallic acid, vanillin, and vanillic acid, with gallic acid being the most useful. There is clearly a great deal of overlapping between the different groups, with just a few scattered outliers. The relatively clear differentiation between rum and

Table 6. Concentration (ppm) of some nonvolatile phenolic compounds in different commercial brands of brandy other than cognac and armagnac

Origin	Brand No.	Gallic acid	Vanillic acid	Syringic acid	Vanillin	Syringaldehyde	Ferulic acid
France	1	5.03	0.29	0.67	0.87	2.16	ND[a]
	2	3.14	tr[b]	tr	0.17	0.21	ND
South Africa	1	2.39	0.32	0.49	0.49	1.26	ND
	2	5.57	0.59	0.70	0.69	2.05	tr
Italy	1	5.49	tr	0.05	0.52	0.18	ND
Bulgaria	1	7.66	0.49	1.00	1.16	2.54	ND
Spain	1	4.27	0.16	0.27	1.12	1.00	ND
Soviet Union	1	2.38	ND	tr	0.43	0.51	ND
Greece	1	1.59	ND	ND	0.97	1.00	ND
Mixture of brandy distillates originating from different countries		4.74	0.24	0.29	0.57	0.84	ND

[a] ND = none detected.
[b] tr = trace, <0.01 ppm.

Table 7. Concentration (ppm) of some nonvolatile phenolic compounds in different commercial brands of rum

Origin	Brand No.	Gallic acid	Vanillic acid	Syringic acid	Vanillin	Syringaldehyde	Ferulic acid
				Dark Rum			
Martinique	1	2.10	tr[a]	0.10	0.30	0.26	ND[b]
	2	2.29	0.31	0.64	0.64	1.33	ND
Caribbean	1	0.19	ND	tr	0.31	0.22	ND
Haiti	1	ND	2.94	4.77	3.17	8.73	ND
Guadeloupe	1	2.71	1.13	2.63	1.77	4.45	tr
Jamaica	1	ND	ND	ND	ND	ND	ND
Mixture of Finnish sulfite spirit and Martinique Rum	1	ND	ND	ND	ND	ND	ND
				White Rum			
Martinique	1	ND	ND	ND	tr	ND	ND
Caribbean	1	ND	0.37	0.76	0.91	1.88	ND
Guadeloupe	1	ND	ND	ND	ND	ND	ND
Puerto Rico	1	ND	tr	0.18	ND	0.26	ND
	2	tr	tr	0.29	tr	0.51	ND
Cuba	1	ND	ND	ND	ND	ND	ND
Demerara	1	ND	ND	ND	ND	ND	ND
Brazil	1	ND	ND	ND	ND	ND	ND

[a] tr = trace, <0.01 ppm.
[b] ND = none detected.

brandy is mainly due to the phenol-poor white rums. Whiskies appear to lie closer to the rums than to the brandies. In general, however, no firm conclusions can be drawn from these results.

Using discriminant analysis to classify the spirit samples into their respective groups (Table 9) was more successful for the whiskies and brandies (more than 50% correct) than for the rums (about one-third correct). In addition to the correct assignments, there were some samples that did not clearly belong to any group, and these were collected in a special group. There were also some completely false assignments because of similarities in the contents of nonvolatile phenolic compounds.

Conclusions

Liquid chromatography is well suited to the analysis of nonvolatile phenolic compounds in alcoholic beverages. Most matured spirits contain a variety of these components; white rums are exceptional in their low content. Cognacs contain more gallic acid than do the other bev-

Table 8. Determination (mg/L) of phenols in standard solution and recovery (mg/L) of phenolic compounds added to whisky

Compound	Concn in std soln	Found[a]	Amt added to whisky	Rec.
Gallic acid	5.00	5.22 ± 0.13	2.65	2.66
			5.31	5.24
Vanillic acid	5.00	5.04 ± 0.08	2.57	2.01
			5.14	4.03
Syringic acid	5.00	5.10 ± 0.09	2.58	2.21
			5.16	4.43
Vanillin	5.00	5.12 ± 0.08	2.58	2.70
			5.16	5.14
Syringaldehyde	5.00	4.95 ± 0.07	2.50	2.60
			5.00	4.91
Ferulic acid	5.00	4.98 ± 0.09	—	—

[a] Average of 6 independent determinations ± standard deviation.

Table 9. Classification of whiskies, rums, and brandies into their respective groups according to concentration of nonvolatile phenolic compounds

Beverage	No. of brands	No. of correct classifications on the basis of Mahalanobis' distance[a]	No. of uncertain classifications on the basis of Mahalanobis' distance[b]	No. of correct classifications on the basis of posterior probability[c]	No. of uncertain classifications on the basis of posterior probability[d]
Whisky					
Scotch	15	9	0	9	3
Irish	2	2	0	2	0
United States	4	2	1	2	1
Canadian	1	1	0	1	0
Japanese	5	3	0	3	2
Spanish	1	0	1	1	0
Argentina	2	0	0	1	1
New Zealand	2	2	0	2	0
India	1	1	0	1	0
Total	33	20 (61%)	2 (7%)	22 (67%)	7 (21%)
Dark rum	7	5	2	2	2
White rum	8	8	0	3	5
Total	15	13 (87%)	2 (13%)	5 (33%)	7 (47%)
Cognac	11	8	2	5	5
Brandy	10	8	0	6	2
Total	21	16 (73%)	2 (10%)	11 (52%)	7 (33%)

[a] Mahalanobis' distance is clearly the shortest.
[b] Mahalanobis' distance in the correct group is nearly equal that in the other group.
[c] Posterior probability is clearly the greatest.
[d] Posterior probability in the correct group is nearly equal that in the other group.

erages, and U.S. Bourbon whisky contains more syringaldehyde. In other respects, the similarities are striking. The differences between the contents of nonvolatile phenolic compounds in different beverages are too small to allow the classification of beverages into their correct groups with any great confidence.

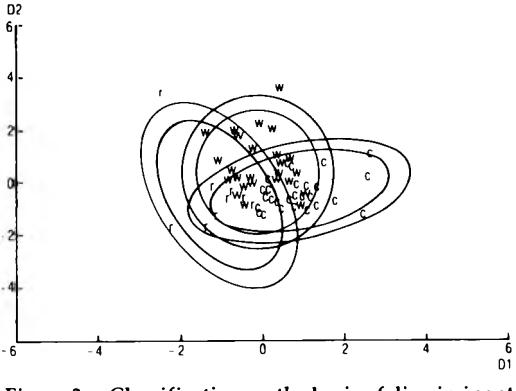

Figure 3. Classification on the basis of discriminant functions calculated from concentrations of phenolic compounds in whisky (w), rum (r), and brandy (c). Inner ellipses cover 95% of samples and the outer cover 99%. Coordinates of centroids are: whisky (−0.100; 0.425), rum (−1.054; −0.481), and brandy (0.910; −0.324). Total discriminating power of variables, $F_{16,124} = 6.98$, is significant at the 1% level.

Acknowledgments

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EXTRANEOUS MATERIALS

Reliability of Analyses for Indigenous Insect Fragments in Ground Paprika

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Variation in cleanliness (light filth) determinations within and between laboratories for paprikas with indigenous insect fragments was significantly greater than previously reported for paprika with spiked insect fragments.

New methods (1) for the analysis of cleanliness (light filth) of capsicums were published in the 13th edition of *Official Methods of Analysis* of the AOAC. A collaborative study reported in the *Journal* of the AOAC (2) indicated that the new method for paprika gave cleaner filter papers and higher recoveries than the old method. In that collaborative study, samples of ground paprika were spiked with rodent hair fragments and insect fragments of specific shapes and sizes so that recovery could be determined. The repeatability and reproducibility coefficients of variation were reported at 6.3 and 6.5%, respectively, for insect fragments.

The American Spice Trade Association's (ASTA) technical committee initiated a study with the new paprika method, for the purpose of determining repeatability and reproducibility for paprika with indigenous (nonspiked) fragments. Three different samples of ground paprika were carefully mixed and divided and sent to the 3 independent laboratories that analyze for cleanliness most of the paprika imported into the United States. Duplicate analyses by the current AOAC method (1) were requested for each sample.

Results and Discussion

In Table 1, the reproducibility (between laboratories) coefficient of variation (CV) was 32.6% compared with 6.5% reported for the spiked samples (2). The repeatability (within laboratories) CV was 20.6% compared with 6.3% for the spiked samples. The repeatability CV includes not only error due to the laboratory's ability to produce the same results on the same material, but also any inherent variation that exists due to the nonuniform distribution of the insect fragments in carefully mixed and divided samples. With spiked samples, nonuniform distribution does not contribute to the variation. Other possible reasons for higher repeatability CV for paprika with nonspiked fragments include: (*1*) Smaller fragments may be more difficult to extract and separate. Spiked insect fragments were approximately 0.5 mm long (0.25 sq. mm); those normally found in ground paprika range from 0.01 to 0.3 mm (0.0001 to 0.09 sq. mm). (*2*) Fragments dehydrated and ground with the paprika may be more difficult to extract from the vegetable matter than added or spiked fragments. (*3*) Smaller insect fragments are more

Table 1. Repeatability and reproducibility for analysis of indigenous insect fragments in paprika

	Sample 1		Sample 2		Sample 3		
Lab.	A	B	A	B	A	B	All 3 samples
1	16	30	60	49	49	42	
2	59	55	59	44	29	31	
3	31	16	56	62	26	48	
Mean	34.5		55.0		37.5		42.3
σ_0^2							75.3
σ_t^2							−58.7
σ_{ls}^2							172.5
σ_x^2							189.1
σ_0 (repeatability)							8.7
σ_x (reproducibility							13.8
CV within labs, %							20.6
CV between labs, %							32.6

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difficult to identify, leading to more variation between laboratories.

Conclusions and Recommendations

The repeatability CV in this study with indigenous insect fragments was more than 3 times that of the 1977 study using spiked samples. The official AOAC method is easier to perform and does result in cleaner plates and higher recoveries than the method it replaces. It is suggested that a future study include determination of repeatability and reproducibility on nonspiked samples as well as spiked samples.

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OILS AND FATS

Improved Lipoxygenase Method for Measuring *cis,cis*-Methylene Interrupted Polyunsaturated Fatty Acids in Fats and Oils

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An improved lipoxygenase method is described for the determination of *cis,cis*-methylene interrupted polyunsaturated fatty acids in fats and oils. The methodology is designed to overcome mechanical problems which in the past may have contributed to poor precision. This method provides improved repeatability which is imperative for assay methods used for quality control and other purposes.

Voluntary labeling of foods in relation to fat, fatty acid, and cholesterol content (1) has increased the need for reliable data and methodology for determining the content of specific fatty acids in foods. Determination of *cis,cis*-methylene interrupted polyunsaturated fatty acids (PUFA) (2) has always been one of the most difficult analyses to perform with the desired repeatability (standard deviation within a laboratory) and reproducibility (standard deviation between laboratories). Studies in our laboratories and elsewhere have confirmed the MacGee lipoxidase enzymatic method for the determination of PUFA (3) to be the procedure of choice. The current United States regulation has specified the Canadian Food and Drug Directorate Method FA-59 (4) (a modification of the MacGee method) for determination of *cis,cis*-methylene interrupted PUFA. Two modifications of the MacGee procedure have also been compared in an AOAC collaborative study (5). The methods evaluated were one proposed by the Food and Drug Administration (6) and one used by industry (7, 8). Over all the oil samples studied, the 2 methods gave similar results and there was no overall statistical difference in the performance. The repeatabilities and their respective average coefficients of variation (7.8 and 6.7) indicate that both methods are basically sound but that further study and modifications are needed. The reproducibility results for coefficient of variation (10.2 and 9.1 for the 2 methods) also supported the need for further study to improve the precision. (Note: Method II in this collaborative study became an official AOAC method (9).)

The present paper summarizes studies in our laboratory and presents a method which uses increased amounts of enzymes and saponification reagents but no solvent for samples. This procedure has been tested for durability and provides improved repeatability which we believe is imperative for assay methods used for regulatory control.

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Experimental

Reagents

(**a**) *Potassium borate buffer.*—1.0M, pH 9.0. Dissolve 124.0 g H_3BO_3 and 50.0 g KOH in ca 1600 mL water by stirring and heating. Cool to room temperature and adjust to pH 9.0 with 1.0N HCl or 1.0N KOH, as required. Dilute to 2 L with water and mix.

(**b**) *Dilute potassium borate buffer.*—0.2M, pH 9.0. Dilute 400 mL 1.0M buffer, (**a**), to 2 L with water and mix.

(**c**) *Lipoxygenase solutions.*—(*1*) *Active solution.*—Dissolve 50 mg lipoxygenase, from soybean, Type I, No. L-7127 (Sigma Chemical Co.) in 500 mL ice-cold dilute borate buffer, (**b**). Store in refrigerator. (*2*) *Inactivated solution.*—Transfer 200 mL active enzyme solution, (*1*), to 250 mL volumetric flask and hold in boiling water 30 min. Store in refrigerator.

(**d**) *Potassium hydroxide solutions.*—(*1*) *10.0N.*—Dissolve 330.0 g KOH in ca 330 mL water. Cool to room temperature. Dilute to 500 mL and mix. Store in polyethylene container. (*2*) *Alcoholic solution.*—1.0N. Transfer 10 mL 10.0N solution, (*1*), to 100 mL volumetric flask. Dilute to volume with alcohol. Prepare fresh daily.

Apparatus

(**a**) *Double beam spectrophotometer.*—Beckman Acta MIV, or equivalent, equipped with matched quartz 10 mm cells.

(**b**) *Desiccator.*—Equipped with nitrogen inlet and outlet (No. 108-225, Curtin Matheson).

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Table 1. Outline of procedures for measuring *cis,cis*-methylene interrupted PUFA in fats and oils

Procedure component	AOAC method (9)	Proposed method
Sample wt, mg	100	100
Solv. for samples & stds	hexane	samples: none stds: ethanol
Aliquot of alc. KOH	1 mL of 0.5N	5 mL of 1.0N
Aliquot of HCl	1 mL of 0.5N	5 mL of 1.0N
Concn of enzyme		
Stock soln	10 mg/10 mL	50 mg/500 mL
Working soln	0.10 mL of 2 mg/10 mL	9.0 mL of 1 mg/10 mL
Aliquot of sample soln oxidized with enzyme	3.0 mL from 100 mL	1.0 mL from 100 mL
Final vol. sample soln	3.1 mL	100 mL

(c) *Circulating water bath.*—Neslab RTE-8, or equivalent, at 15°C.

Preparation of Sample

Accurately weigh ca 100 mg vegetable oil or melted shortening into 25 mL Erlenmeyer flask.

Determination

Add 5 mL alcoholic KOH, (d)(2), to sample in 25 mL Erlenmeyer flask. Cover with tuttle cover to fit flask. Heat contents to boiling on hot plate to dissolve sample. Hold in dark desiccator flushed with nitrogen >4 h or preferably overnight. After saponification is complete, add 5 mL 1N HCl. Solution should be clear at this point. If not, add 1.0N KOH dropwise until solution clears. Transfer entire sample, using hot water, into 100 mL volumetric flask containing 20 mL 1.0M borate buffer, (a). Rinse Erlenmeyer flask with hot water and add rinse to sample. Warm gently on steam bath to dissolve any hard soaps present. Dilute to volume with water and mix. Pipet 1.0 mL saponified solution into each of two 100 mL volumetric flasks. Add 9.0 mL inactivated enzyme solution, (c)(2), and mix well. To remaining volumetric flask, add 9.0 mL active enzyme, (c)(1). Add 10.0 mL water to each flask, mix, and place both flasks in 15°C constant temperature water bath. Blow air into each flask 30 min. Dilute to volume with water and mix. Zero spectrophotometer at 234 nm with blank sample and measure *A* of reacted samples.

Preparation of Standard Curve

Quantitatively weigh 50 mg *cis,cis*-trilinolein, 99% (Analabs, Inc., No. LGL-017) into 25 mL volumetric flask, and dilute to volume with anhydrous ethanol. Pipet 0.5, 1.0, 2.0, 3.0, and 4.0 mL aliquots into separate 50 mL beakers, and evaporate each to dryness in stream of nitrogen. Pipet 5 mL alcoholic KOH, (d)(2), into each beaker. Heat contents to boiling and store in dark desiccator under nitrogen >4 h or overnight. Add 5 mL 1N HCl and mix. If solutions are not clear, add 1.0N KOH dropwise until clear. Transfer each standard solution into separate 100 mL volumetric flasks containing 20 mL 1.0M borate buffer, (a). Dilute to volume with water and mix. Pipet 1.0 mL aliquots of each standard solution into each of two 10 mL volumetric flasks, incubate, and read as in determination. Plot *A* at each level against mg trilinolein in 100 mL.

Calculations

cis,cis-PUFA, % = (mg trilinolein in 100 mL × 10^{-3} g/mg × 100)/(mg sample weight in 100 mL × 10^{-3} g/mg)

Trilinolein, g/100 g product = *cis,cis*-PUFA, %/100

Results and Discussion

The principal differences between the AOAC method (9) and the proposed method are outlined in Table 1. These changes improve the mechanics of the method, eliminate the use of organic solvents for samples other than ethanol for standards, and eliminate the need to handle small volumes of reagents. A more detailed discussion of the modifications is described in the following sections.

Saponification

Samples containing a high concentration of saturated fatty acids require gentle heating of the sample to ensure complete contact with the saponification reagent. Also, alkali used for saponification must be neutralized before the sample is reacted with enzyme. It was observed that after neutralization of alkali with hydrochloric acid, a precipitate sometimes formed and,

Table 2. Effect of enzyme level on corn oil absorbance values and calculated *cis,cis*-PUFA

Enzyme level, mL	Corn oil, g	Absorbance	Calcd, %[a]
1	0.112	0.113	13.4
3	0.1131	0.340	36.7
5	0.1120	0.509	54.9
9	0.1104	0.542	59.3
15	0.1135	0.556	59.3

[a] Nominal % *cis,cis*-PUFA, 59.3%.

if not solubilized, caused low results. This precipitate formation was attributed to solution pH and resulting precipitation of some fatty acids. This problem was easily corrected by using tuttle covers for the Erlenmeyer flasks to minimize solvent loss during heating, and the back-addition of a few drops of 1.0N potassium hydroxide until the solution cleared. The 1.0M borate buffer was then added to the sample and it was made ready for enzymatic oxidation.

Enzymatic Oxidation

Lipoxygenase catalyzes the oxidation by atmospheric oxygen of PUFA which contain at least one pair of *cis*-double bonds separated by a methylene group. Oxidation was carried out at 15°C using a constant temperature water bath to avoid temperature change for the sample. Complete hydroperoxide formation and the enzyme level required was established using corn oil and adding various amounts of the active working lipoxygenase solution described in the method. The absorbance values and calculated *cis,cis*-PUFA shown in Table 2 demonstrate the effect of enzyme level on results. The increased amount of enzyme level selected for use in this method (9 mL) is appropriate to measure "*cis-,cis*-PUFA" level. The absorbance of the oxidized sample solution remained stable for at least 5 h. Since each mole of trilinolein yields 3 moles of linoleic acid, a plot of absorbance vs μg trilinolein yielded a linear graph. When absorbance values are calculated on the basis of the molar extinction and the amount of linoleic acid in the calibration standards, the calculated absorbance values agree well with the experimental values obtained (Table 3).

Table 3. Determination of absorbance values by calculation from molar extinction and experimental

Trilinolein, mg/100 mL	Linoleic acid, mole/L	Absorbance	
		Calcd[a]	Expl
20	6.80×10^{-6}	0.160	0.159
40	13.62×10^{-6}	0.320	0.325
60	20.43×10^{-6}	0.480	0.477
80	27.42×10^{-6}	0.639	0.631

[a] a = 23 472.

Table 4. Standard deviations of various oil lots used as secondary standards

Year	Sunflower oil (63%)[a]	Commercial shortening (23%)[a]
1979[b]	2.39 (10)[c]	1.23 (43)
1979[d]	1.28 (25)	0.96 (26)
	0.62 (11)	0.47 (10)
1980[d]	1.07 (8)	0.40 (8)
	1.13 (9)	0.61 (9)
1981[d]	1.21 (27)	0.48 (27)

[a] Nominal %, *cis,cis*-PUFA values.
[b] Data obtained before method improvement.
[c] Number of replicates.
[d] Data obtained after method improvement.

Repeatability

Table 4 lists the standard deviations of replicate analyses on a variety of oil and shortening samples analyzed in our laboratory over a period of years. The results obtained in 1980 and 1981 represent the repeatability achieved using the proposed method described herein. The relative standard deviation or coefficient of variation measured (~2%) represents a significant improvement over the 6.7% obtained in the 1978 AOAC collaborative study (5). To further test the durability of this modified method over time, samples of sunflower oil and a commercial shortening were analyzed as sample pairs by different analysts as part of an Internal Precision Program. These data points were obtained in about 80–90 days over each year's time and again yielded an RSD of 1.9%. For reference, this equates to a standard deviation of about 0.4% for a sample containing 20% PUFA and about 1.1% for a sample containing 60% PUFA. The AOAC collaborative study within-laboratory data (5) would yield standard deviations of 1.46 and 4.38%, respectively.

The difference in the number of replicates is due to the number of times the samples were analyzed in a given year.

Three fats previously analyzed by capillary gas chromatography for *cis,cis*-C18:2 content were measured using the modified procedure. The gas chromatography procedure (D. C. Underwood and R. Houston, private communication) used a Hewlett-Packard 5880 instrument equipped with a flame ionization detector (280°C) and a 50M Silar 10C Quadrex glass cap-

Table 5. Low range *cis,cis*-PUFA samples analyzed by the modified method and capillary GC

Fat sample	*cis,cis*-PUFA Modified method	*cis,cis*-C18:2 Capillary GC
1	8.4	7.8
2	none found	0.7
3	16.9	15.0

illary column. A 1 μL sample of the methyl ester was injected (260°C) using programmed temperature conditions (150°C–190°C, 1.3°/min) and helium carrier gas (0.5 mL/min). The results in Table 5 show good agreement between methods and demonstrate the method's applicability to low range *cis,cis*-PUFA samples.

The modified lipoxygenase method described herein provides a reliable and precise method to be used for the assay of *cis,cis*-methylene interrupted PUFA in fats and oils. The methodology is designed to overcome mechanical problems which in the past may have contributed to poor precision (10). The improved precision achieved and demonstrated should allow extensive use of this method for the analysis of PUFA in various foods.

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MYCOTOXINS

Rapid Thin Layer Chromatographic Determination of Patulin, Citrinin, and Aflatoxin in Apples and Pears, and Their Juices and Jams

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A method is described to determine the mycotoxins patulin, citrinin, and aflatoxins in apples and pears and their juices and jams. The mycotoxins are extracted with a mixture of acetonitrile and 4% aqueous KCl (9 + 1). The extract is cleaned up with water and then acidified, and the toxins are recovered with chloroform and separated by thin layer chromatography. Toxin identity is confirmed with various developing solvents, spray reagents, and chemical reactions, and then quantitated by the limit of detection method. The minimum detectable concentrations of the mycotoxins are patulin, 120-130 μg/kg; citrinin, 30-40 μg/kg; aflatoxin B_1 or G_1, 2-2.8 μg/kg; aflatoxin B_2 or G_2, 2 μg/kg.

The natural occurrence of patulin has been associated with *Penicillium expansum* and other fungal species (1-3). *P. expansum* is a common storage rot organism in apples (4-6), pears, and other fruits, and patulin has been positively identified in apples, pears, apricots, and apple juice (6-11). Citrinin was originally isolated from *P. citrinum* (12-15) and other fungal species. Harwig et al. (6) have also isolated strains of *P. expansum* from natural rots of apples that were capable of synthesizing citrinin and patulin; they also reported (16) the production of citrinin in tomatoes inoculated with *P. expansum* (incubated at 25°C). *P. expansum* has been shown to produce citrinin and patulin in fermented sausage (14). Ciegler et al. (17) studied the production and biological activity of patulin and citrinin from *P. expansum*.

Aflatoxins was originally isolated from *Aspergillus flavus* and *A. parasiticus* (18, 19). Hanssen and Jung (7) studied the natural contamination by patulin and aflatoxins in apples and other fruits. Burdaspal and Pinilla (10) found aflatoxins, patulin, and citrinin in rotted apples. Frank (20) found patulin in different varieties of apples and pears suffering from brown rot.

The AOAC official method (21) for analysis of patulin in apple juice only has a sensitivity of 20 μg/L. The following method permits the simultaneous analysis of patulin, aflatoxins, and citrinin in apples and pears, and the juices and jams of these fruits. For patulin, the following method is less sensitive than the official method (21); however, the concentrations of patulin found in fruits are normally higher than minimum detectable concentrations in both methods.

METHOD

Apparatus

(a) *Microsyringes.*—50 μL No. 1705 or 25 μL No. 702, point style 3 with repeating dispenser PB 600-1 (Hamilton Co.), or equivalent.

(b) *Electric mixer.*—Moulinex, Braun Minipimer, or equivalent.

(c) *Shaker.*—Wrist or rocker type (Bicasa K30/300 BE38, or equivalent).

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

(e) *Glass tube.*—Amber, pear-shaped, 300 mL. One end narrows to 4.5 × 1 cm id. Narrow section is graduated as accurately as possible to ca 3 mL capacity and marked in 0.1 mL divisions. Other end of tube is cone-shaped ground glass, 29/32 adaptable to rotary evaporator and glass stopper. Glass must be strong enough to withstand vacuum (Figure 1).

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

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

(h) *Ultraviolet lamp.*—Camag standard 29200 or 29230 with a blue-violet filter (UG-5, Schott, GFR) passing 366 and 254 nm UV light, or equivalent. 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) which passes

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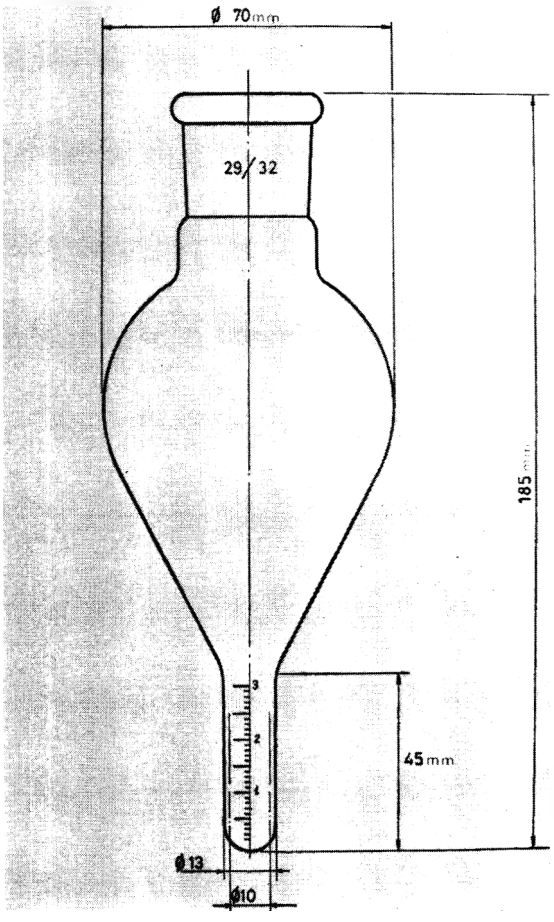

Figure 1. Glass tube used for evaporation of solvent.

only 450 nm light, or equivalent.

(**j**) *TLC plates.*—20 × 20 cm glass, precoated with 0.25 mm Sil G-25HR (Macherey-Nagel and Co., No. 809033, Duren, GFR). Use TLC plate scriber to divide plates into twenty 1 cm strips. Activate plates by heating 30 min at 110°C and store in desiccating cabinet. Use plates at room temperature (22, 23).

(**k**) *TLC plates impregnated with oxalic acid.*—Unactivated, precoated with Sil G-25HR (divided into twenty 1 cm strips) (23, 24). Impregnate with oxalic acid by dipping into 10% oxalic acid solution in methanol for 1–2 min. Air-dry 4 or 15 h at room temperature in horizontal position under hood (dry atmosphere). (For citrinin analysis only.)

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

(**m**) *Other apparatus.*—As described in Ref. 23.

Reagents

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

(**b**) *3-Methyl-2-benzothiazolinone hydrazone (MBTH) hydrochloride monohydrate solution.*—Dissolve 0.5 g $MBTH \cdot HCl \cdot H_2O$ in 100 mL water. Store in refrigerator. Prepare fresh every 3 days.

(**c**) *Sulfuric acid solution.*—H_2SO_4–water (1 + 3).

(**d**) *Trifluoroacetic acid.*—Dilute with benzene or $CHCl_3$ (1 + 1). Prepare fresh daily. (Caution: Benzene and $CHCl_3$ are possible carcinogens.)

(**e**) *Mycotoxin standards.*—Xpectrix International, San Cugat del Valles, Barcelona, Spain. Store in well sealed, aluminum foil-wrapped containers at 4°C.

(**f**) *Mycotoxin standard solutions.*—Prepare with reagent grade $CHCl_3$. Store solutions in well

sealed, aluminum foil-wrapped containers or in amber vials at 4°C. Use solutions at room temperature. (1) *Aflatoxins B_1, B_2, G_1, and G_2.*—Prepare separate 10 μg/mL $CHCl_3$ solutions of each aflatoxin and combine volumes of each solution to prepare working solution that contains 5 μg B_1, 1.4 μg B_2, 5 μg G_1, and 1.4 μg G_2/mL $CHCl_3$. (2) *Patulin.*—100 μg/mL $CHCl_3$. (3) *Citrinin.*—45 μg/mL $CHCl_3$. Check standards as in Ref. 25.

(g) *Developing solvents for TLC.*—Prepare fresh with reagent grade solvents as shown:

1. $CHCl_3$–acetone (176 + 24), (172 + 28), (170 + 30) (22–24)
2. $CHCl_3$–ethyl ether–acetic acid (170 + 30 + 10) (26)
3. $CHCl_3$–acetone–propanol-2 (165 + 30 + 5) (27)
4. Toluene–ethyl acetate–$CHCl_3$–90% formic acid (70 + 50 + 50 + 20) (22–24)
5. Toluene–ethyl acetate–90% formic acid (100 + 90 + 10) (22–24)
6. Benzene–$CHCl_3$–acetone (90 + 80 + 30) (28)
7. Toluene–ethyl acetate (50 + 150) (26)
8. Toluene–ethyl acetate–$CHCl_3$ (80 + 70 + 50)
9. Ethyl ether–cyclohexane (150 + 50) (26)
10. $CHCl_3$–ethanol (170 + 30) (22, 23)
11. $CHCl_3$–methanol (185 + 15)
12. Toluene–ethanol (100 + 100) (26)
13. Toluene–ethyl acetate–$CHCl_3$ (100 + 50 + 50) (26) (34)
14. Toluene–$CHCl_3$–acetone (40 + 140 + 20)
15. Toluene–$CHCl_3$–acetone–90% formic acid (30 + 150 + 20 + 4) (23, 24)
16. Toluene–ethyl acetate–$CHCl_3$–90% formic acid (90 + 45 + 50 + 5) (23, 24)
17. Toluene–$CHCl_3$–ethyl acetate (25 + 145 + 30) (23, 24)
18. Toluene–$CHCl_3$ (10 + 190) (23, 24)
19. Ethyl ether–hexane–ethyl acetate–90% formic acid (70 + 90 + 40 + 2) (24)
20. $CHCl_3$–methanol–hexane (128 + 2 + 70) (29)
21. Ethyl ether–hexane–ethyl acetate–90% formic acid (50 + 100 + 50 + 1)
22. Toluene–ethyl acetate (65 + 135)
23. Toluene–ethyl acetate–$CHCl_3$–90% formic acid (105 + 45 + 48 + 3)
24. Toluene–ethyl acetate–90% formic acid (100 + 95 + 5) (34)

Extraction and Cleanup

For apples, pears, and their jams, blend with electric mixer to homogeneous paste. Weigh 60 g sample (for juices, take 60 mL) and place in 500 mL amber, glass-stopper Erlenmeyer flask. Add 180 mL acetonitrile and 20 mL 4% aqueous KCl, 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 200 mL amber separatory funnel (PTFE stopper and stopcock). Add 30 mL water and shake 30 s. Add 40 mL chloroform, stopper, and shake 15–20 s. Let separate into layers. Filter lower layer (chloroform–acetonitrile) through 9 or 11 cm MN 640w paper filled to top with anhydrous sodium sulfate. Collect filtrate in pear-shape glass tube. To remaining aqueous layer, add 2.0 mL 1N HCl and shake. Add 20 mL chloroform, stopper, and shake 20 s. Let separate into layers. Filter lower (chloroform) layer through anhydrous sodium sulfate used previously and add to filtrate already collected in pear-shape glass tubes. Repeat extraction of aqueous layer 3 times with 10 mL $CHCl_3$ each and filter each extract as before.

Place pear-shape glass tube in rotary evaporator and evaporate contents to 2 mL at 50–55°C (water bath) with vacuum (depression = 0.6–0.7 kg/cm^2). Cool tube and wash walls with three 1 mL portions of $CHCl_3$, carrying liquid to narrow graduated part. Evaporate to 0.1–0.2 mL (Caution: Do not evaporate to dryness) at 50–55°C with vacuum (depression = 0.7–0.85 kg/cm^2). Whenever vacuum is interrupted, nitrogen gas must be added. Cool, and add $CHCl_3$ to 1 mL division of narrow graduated section. Stopper and shake vigorously on tube shaker. Final extract volume for TLC will be 1.0 mL (1.0 mL $CHCl_3$ solution = 18 g extracted sample or 18 mL for juices). Place extract solution in amber minivial with Teflon stopper (22, 23).

Thin Layer Chromatography

Patulin (plates A, B, and C).—On imaginary line 4 cm from bottom of plate (j), place following spots at 1 cm intervals, in middle of strip: two of 10 μL extract solution (do not use strips at edges of plate). To one spot corresponding to 10 μL, superimpose 3 μL patulin standard solution (internal standard). On a clear strip, place 3 μL patulin standard solution (external standard). Take care that diameter of spots, once total volume has been deposited, does not exceed 2–2.5 mm (dry spots with nitrogen gas). Draw transverse line on TLC plate 12 cm from imaginary line as solvent stop.

Place in TLC separating chamber ca 200 mL developing solvent, ca 1.4–1.5 cm, cover, and let stand 5 min. Then insert TLC plate into chamber in exactly vertical position with silica gel

surface 3–5 cm from front wall of chamber. Cover and develop in unsaturated chamber until solvent front reaches height indicated before. Develop plate A with developing solvent 22 or 23; confirm with plates B and C by developing with solvents 8 and 9 or 24, respectively.

Remove plate from tank and let dry in dark hood. Force final drying with gentle current of nitrogen gas or moderately hot air (not more than 35–40°C). Spray TLC plate with MBTH solution until layer appears wet. Heat 15 min at 130°C (21), and observe under visible light for reflection and transparency at same time; patulin shows yellow spot. Then observe under 366 nm UV light; patulin shows yellow-brown fluorescent spot. Compare sample chromatogram pattern with that of internal standard and external standard (same R_f value and color identical to patulin standard in the 3 chromatoplates). Spray TLC plate with 90% formic acid–water (2 + 98) until layer appears wet (spray generously); leave at room temperature 30 min or more and observe under 366 nm UV light. Patulin shows yellow-orange fluorescent spot and improved visualization against background. Under 254 nm UV light, yellow-orange spot of patulin is visible but less intense than previously (34).

Other developing solvents for patulin: Nos. 1, 7, 10, 11, 12, 13. Good results are obtained with developing solvents 8 and 9 to which 1 mL 90% formic acid has been added, developing solvents 1, 10, 11, 12, 13, to which 0.5 mL 90% formic acid has been added (34).

Aflatoxins G_2, G_1, B_2, B_1 (plates D, E, and F).—See patulin section for spotting, developing, etc., but use aflatoxin working solution. Develop plate D with developing solvent 1. Observe under 366 nm UV light. Aflatoxins B_1 and B_2 show blue fluorescent spot; aflatoxins G_1 and G_2 show blue-green fluorescent spot. Spray plate with H_2SO_4–water (1 + 3); observe under 366 nm UV light (22, 23, 26). Aflatoxins show yellow fluorescent spot. Order of appearance from lesser to greater R_f is aflatoxin G_2, G_1, B_2, B_1.

Confirmation (plates E and F).—Treat both plates with trifluoroacetic acid (23, 30) and develop with solvents 2 and 3, respectively. Observe under 366 nm UV light (22, 23, 30). Spray with H_2SO_4–water (1 + 3) and observe under 366 nm UV light (22, 23, 26, 30). Compare sample with internal and external standards.

Other developing solvents for aflatoxins: Nos. 4, 5, 6. Note: Confirmation with trifluoroacetic acid is also possible with developing solvents 1, 4, 5, 6.

Citrinin (plates G, H, and I).—Use TLC plates impregnated with oxalic acid. See patulin section for spotting, developing, etc., but use citrinin standard solution. Develop plates G, H, and I with developing solvents 20, 19, and 16, respectively. Observe under 366 and 254 nm UV light; citrinin shows yellow fluorescence that is less intense under 254 nm UV light (22–24, 26). Expose plates G and H to NH_3 vapor for 10–15 s (22–24) and observe under 366 and 254 nm UV light (34); yellow fluorescence of citrinin disappears. Spray plate I with aluminum chloride solution, heat 5 min at 105°C, cool, and observe under 366 and 254 nm UV light (22–24); citrinin shows sky blue fluorescence, more intense under 366 nm UV light. Expose plate I 10–15 s to NH_3 vapor and observe under 366 and 254 nm UV light; the sky blue fluorescence of citrinin does not completely disappear, at least in spots containing 0.1–0.2 μg/spot. Compare sample with internal and external standards. Other developing solvents for citrinin: Nos. 14, 15, 17, 18, 21.

Quantitative Analysis

Once toxins have been identified on all TLC plates by comparison of extract spots with internal and external standards, proceed to quantitative analysis for positive samples.

Chromatograph a series of dilutions from sample extract to determine lowest dilution at which mycotoxin can still be detected. From lower limit of detection (sensitivity on TLC plates, μg/spot) and dilution factor, calculate quantity of mycotoxin (22, 23, 34).

Relate volume of spot for which mycotoxins can be verified with sensitivity on TLC plates for mycotoxin in question (see Table 1). Interpolate between 2 volumes if necessary.

$$\mu g \text{ Mycotoxin/kg or L product} = (D \times F \times 10^6)/(a \times W)$$

where D = sensitivity on TLC plates (see Table 1), μg/spot of corresponding mycotoxin; F = total factor of dilution in mL; a = volume, μL, corresponding to spot of least mycotoxin detectable on TLC plate; W = weight of sample, g, or volume of sample, mL (see text = 60 g or 60 mL).

Results and Discussion

Table 1 gives the sensitivity on the TLC plate (limit of detection, μg/spot) of corresponding mycotoxins. Recoveries (%) and minimum detectable concentrations (μg/kg) of mycotoxins added to various products are given in Table 2, based on 5 determinations (each value is the mean of 5 samples). The samples of juices and

Table 1. Sensitivity (limit of detection, μg/spot) on TLC plate for 6 mycotoxins in pure solution and in extracts of various products

Mycotoxin	Visualization	Soln of std, μg	Std toxin added to 10 μL product,[a] μg
Patulin	MBTH/visible	0.05	0.05–0.07
Patulin	MBTH/UV 366 nm	0.01	0.01–0.02
Citrinin	UV 366 nm	0.0009	0.001–0.002
Citrinin	$AlCl_3$/UV 366 nm	0.0009	0.001–0.0015
Aflatoxin B_1 or G_1	UV 366 nm	0.00035	0.00035–0.0004
Aflatoxin B_1 or G_1	H_2SO_4/UV 366 nm	0.00020	0.00020–0.00025
Aflatoxin B_2 or G_2	UV 366 nm	0.00030	0.00030–0.00040
Aflatoxin B_2 or G_2	H_2SO_4/UV 366 nm	0.00020	0.00020–0.00025

[a] Product = extracted sample solution of the following products: apples, pears, and juice and jam of apples and pears.

jams analyzed did not contain preservatives.

Either natural or artificial direct light should be avoided during analysis. Working temperature during plate development should be 22–24°C. Once the analysis is begun, steps should not be delayed, especially quantitation by the detection limit method; quantities of mycotoxin are low, and significant losses can occur.

For the patulin reaction with MBTH, observation of the TLC plate while hot improves the visualization of this mycotoxin (31). The standard should not be stored as a thin film after evaporation because of decomposition. If TLC is not performed the same day, the solution should be frozen to avoid evaporation of solvent (21).

Burdaspal and Pinilla (10) found patulin in about 53% of samples analyzed (about 104) of naturally rotted apples, with concentrations between 1 and 250 mg/kg. In naturally rotted pears, they found patulin in about 33% of samples analyzed (about 24) with concentrations between 0.9 and 10 mg/kg. Aflatoxin B_1 and citrinin were found in one apple. They inoculated *P. expansum* in several apples, and after incubation and analysis, found patulin and citrinin (1.2 mg/kg). Concentrations of patulin found in naturally rotted apples were as high as 136 mg/kg fruit (6). Patulin was found at 1.0 mg/L in one of the 12 sample apple juices analyzed (11). In 37% of 136 samples of apple juice, patulin was detected at levels between 40 and 440 μg/L, with an average of 69 μg/L (11). Burdaspal and Pinilla (10) detected patulin in 50% of 12 samples of naturally rotted apricots at levels of 2–13 mg/kg and also in rotted peaches at levels of 20 mg/kg. Concentrations of patulin in apple juice (20–120 μg/L) were lower (2).

Frank (20) studied different varieties of apples and pears suffering from brown rot and found patulin in about 50% of samples analyzed (about 120). Levels of patulin as high as 1 g/kg rotten material were found 2–3 days after the fruit was removed from 5 months of cold storage (22). The author also found the following occurrences of patulin in apple juice (20): commercial apple juice, contamination in 61% of samples analyzed (about 13), maximum level 300 μg/L; commercial apple cider, contamination in 4% and 100% of samples analyzed (about 95 and 5 samples, respectively), maximum levels 25 and 45 ppm, respectively; other commercial apple cider, 60% of samples contaminated (about 15), maximum level 0.3 ppm; commercial sweet apple cider, 0.8% with contamination (12 samples analyzed), maximum level 1 ppm; commercial sweet apple cider (unfermented apple juice), one sample analyzed, maximum level 1 ppm.

We found patulin in 66.6% of samples analyzed (about 30) of naturally rotted apples at levels between 0.8 and 100 mg/kg. About 20 samples were contaminated with patulin, 5 samples were contaminated with aflatoxin B_1 and citrinin at 20–200 μg/kg and 300–3000 μg/kg, respectively, and 3 samples were contaminated with citrinin at 1000–2500 μg/kg.

The method described is rapid and sensitive; 8 samples can be analyzed on each TLC plate. Burdaspal and Pinilla (10) used the method (22–24) for determining patulin, aflatoxins, and citrinin in fruits and found it preferable (10) to the Sommer et al. (32) and Wilson and Nuovo (33) methods. With the method described here, Ligia Martins (unpublished) found patulin in 53.8% of samples analyzed (about 13) of naturally rotted apples, at 200–4100 μg/kg; about 7 samples

Table 2. Recoveries (%) and minimum detectable concentrations (μg/kg) of mycotoxins added to various products [a]

Mycotoxin	Added to apples, μg/kg					Added to pears, μg/kg					Min. detectable concn, μg/kg
	20	80	200	300	500	1000	20	80	200	300	500	1000	
Patulin	—[b]	—	90	91	93	95	—	—	89	92	94	94	120–128
Citrinin	—	87	90	92	93	96	—	89	91	94	94	94	30–39
Aflatoxin B_1 or G_1	96	98	99	100	100	100	96	97	99	100	99	100	2–2.5
Aflatoxin B_2 or G_2	96	97	99	99	100	100	97	97	99	100	100	100	2
	Added to juice, μg/kg						Added to jam, μg/kg						
Patulin	—[b]	—	89	90	93	94	—	—	90	91	93	93	125–130
Citrinin	—	86	89	91	94	93	—	87	89	90	95	95	35–40
Aflatoxin B_1 or G_1	97	97	98	100	99	100	96	98	98	99	99	99	2–2.8
Aflatoxin B_2 or G_2	96	98	98	99	100	100	97	97	97	98	100	100	2

[a] Each value is the mean of 5 samples.
[b] Not detected.

were contaminated with patulin, and 3 samples were contaminated with citrinin at 300–1700 μg/kg. In 2 analyses of naturally rotted pears, one sample contained 1000 μg/kg of patulin.

As far as quantitative analysis is concerned, we believe that the method described is more accurate than the method of comparing different intensities of standard, because it is easier for the human eye to detect the least mycotoxin spot seen than to compare intensity of that spot with the same intensity of standard.

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Survey of Vomitoxin Contamination of 1980 Ontario White Winter Wheat Crop: Results of Survey and Feeding Trials[1]

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During the harvesting of the 1980 Ontario white winter wheat crop, producers noticed pink discoloration on kernels; this was attributed to *Fusarium* mold. Grain elevator and boatload samples were analyzed for mycotoxin contamination. Vomitoxin levels up to 8 ppm were detected in samples from southwestern Ontario. Other suspected mycotoxins were either nondetectable or present in trace amounts. *Fusarium*-contaminated wheat and clean wheat were added to swine and poultry diets for feeding trials. Feed refusal and decreased weight gains were observed in pigs fed diets containing 0.3 and 0.7 ppm vomitoxin, but there was no vomiting or other ill effects. Adult roosters and laying hens fed diets containing vomitoxin levels similar to those of the pig diets did not show any overt toxicological effects. Chemical analysis of suspected field cases of vomitoxin-contaminated feed did not reveal high vomitoxin levels.

Reports of feed/food refusal and vomiting in animals and humans date as early as 1916 (1–5). Emesis was observed in swine after ingesting moldy corn and "scabby" grains (3, 6, 7), but swine refused corn acceptable to cattle and poultry (8). In 1973, Vesonder et al. (9) isolated vomitoxin from *Fusarium*-contaminated corn and demonstrated that the toxin produced emesis in swine.

Vomitoxin is a mold metabolite found in corn and cereal grains contaminated in the field by *Fusarium graminearum* Schwabe teleomorph *Gibberella zeae* (Schw.) Petch (10–15). The reddish mold usually begins growing at the ear tip, a condition commonly referred to as "pink ear rot." In the field, infection of corn by *G. zeae* is favored by low temperatures with concomitant high moisture conditions. Once vomitoxin is produced by the mold, it persists and cannot be disguised with feed additives, nor does it disappear with time (8).

In July 1980, Ontario farmers harvesting their white winter wheat crop noticed sprouting and pink coloration on the kernels. Chemical analysis confirmed the presence of 2-deoxynivalenol, or vomitoxin. This paper reports the results of a survey of the 1980 Ontario white winter wheat crop for vomitoxin and other mycotoxins. Exploratory feeding trials were carried out at the Animal Research Center to determine the effects of feeding low levels of vomitoxin to swine and poultry. In addition, several farms were visited where vomitoxin contamination was the suspected cause of vomiting and feed refusal in livestock.

Experimental

Sample Collection and Preparation

The Canadian Grain Commission submitted 41 wheat samples, representing individual producer shipments, to Agriculture Canada for chemical analyses. Thirty-six samples, representing boatloads or partial boatloads, of grain loaded at terminal or export grain elevators were also forwarded for analysis.

The representative samples (5 kg) were delivered unground and immediately stored at 4°C. Before analysis, all samples were ground in a hammer mill to pass a 2 mm screen and mixed thoroughly; 600 g representative samples were taken.

Thin Layer Chromatography (TLC)

Suspected samples were analyzed for the mycotoxins ochratoxin A, zearalenone, sterigmatocystin, aflatoxins (B_1, B_2, G_1, G_2), and patulin by using the acidified acetonitrile extraction—TLC method described by Stoloff et al. (16). Citrinin,

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Table 1. GC-MS analysis of trimethylsilyl derivatives of trichothecenes [a]

Mycotoxin	Retention time, s	Ions monitored, m/z
Vomitoxin	177	512, 497, 235
DAS	214	378, 350, 290
Neosolaniol	318	436, 350, 193
T-2	436	436, 350, 290
HT-2	444	466, 377, 347

[a] Criteria used for positive identifications: all 3 ions characteristic of a given mycotoxin rose and fell simultaneously; they had the same relative abundance as the pure standard material; and the retention time was identical to the standard. Quantitation was carried out by comparison of peak areas of the sample to those of pure standard.

penicillic acid, rubratoxin, luteoskyrin, T-2 toxin, and diacetoxyscirpenol were also included in the TLC standard.

Reference standards for thin layer chromatography were purchased as follows: ochratoxin A, citrinin, and luteoskyrin (Supelco Inc., Bellefonte, PA); T-2, aflatoxins B_1, B_2, G_1, G_2, patulin, diacetoxyscirpenol, rubratoxin, sterigmatocystin, and penicillic acid (Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178); and zearalenone (Myco-Lab Co., Chesterfield, MO 63017).

Derivatization and Determination of Trichothecenes

Twenty g samples were extracted in 500 mL Erlenmeyer flasks by shaking 1 h with 250 mL methanol–water (1 + 1) on a wrist-action shaker (17) and filtered directly into 1 L separatory funnel. The residue was washed with two 50 mL portions of methanol–water. The filtrate and washings were combined and extracted with two 200 mL portions of methylene chloride–ethyl acetate (1 + 1). The resultant extracts were combined and dried over 40–50 g anhydrous sodium sulfate, filtered into a round-bottom flask, and evaporated to dryness. If water residues remained, the sodium sulfate drying procedure was repeated. The residue was transferred to a 15 mL graduated centrifuge tube with two 3 mL portions of methylene chloride–ethyl acetate and evaporated to dryness. Anhydrous pyridine (900 μL), 50 μL TSIM (*N*-trimethylsilylimidazole), and 50 μL TMCS (trimethylchlorosilane) were added and maintained at 60°C for 1 h on a block heater with occasional shaking; samples were then ready for analysis via gas chromatography–mass spectrometry (GC/MS).

TSIM and TMCS silylation reagents were obtained from Chromatographic Specialties, Brockville, Ontario. Vomitoxin (2-deoxynivalenol) was obtained from Myco-Lab Co.; DAS (diacetoxyscirpenol) and T-2 toxin from Applied Science Laboratories, PO Box 440, State College, PA 16801; and HT-2 from Sigma Chemical Co. Stock solutions (1 mg/mL) were prepared in ethanol with warming and stored in the freezer. After evaporation of 20 μL stock solution, 2 mL toluene–acetonitrile (95 + 5) was added to give 10 μg/mL working solutions. Silyl derivatives were prepared as described above.

A combined gas chromatography–mass spectrometry (Finnigan Model 4000 GC-MS system equipped with an INCOS data system, Finnigan Corp., Sunnyvale, CA) was used. The mass spectrometer was operated in the multiple ion monitoring mode for maximum sensitivity and reasonable specificity. The GC column was 1 m × 2 mm (id) glass, packed with 3% SE-30 ultra phase on 80–100 mesh Chrom 705. The column was held at 220°C for 3 min, programmed from 220 to 260°C at 10°/min, and held at 260°C for 2 min. Table 1 shows retention times and the characteristic ions monitored for the 5 mycotoxins analyzed under the above conditions. The lower limit of detection was 0.05 ppm.

Measurement of Fusarium-Staining

The percent *Fusarium*-staining in wheat samples was estimated by taking 500 g aliquots of wheat, weighing those kernels which appeared "stained" or contaminated with pink mold, and expressing results on a percent (%) by weight basis.

Feeding Trials

Swine.—A preliminary study was conducted with 18 Yorkshire gilts fed one of 3 grower diets containing 70% wheat: vomitoxin-contaminated wheat, clean wheat, or a mixture (50 + 50, w/w) of these 2 wheats. The barley–wheat–soybean diets were otherwise identical and met or exceeded NRC (1979) recommended nutrient requirements (18). Pigs were housed 3 to a pen and fed ad libitum; 2 pens of pigs were fed each diet. Feed consumption was determined daily and individual body weights were recorded every 3 days. At the end of the 21-day feeding

Table 2. Analysis for vomitoxin in samples from 1980 Ontario white winter wheat crop—producers' samples

Fusarium stained kernels, %[a]	Numerical grades (Nos. 2–5 CE)[b]		Canada Feed grade		Ungraded	
	No. of Samples	Vomitoxin, ppm	No. of samples	Vomitoxin, ppm	No. of samples	Vomitoxin, ppm
≤0.10	6	ND[c]	12	ND	3	ND
0.11	—	—	1	0.32	—	—
0.12	—	—	4	2-ND, 0.77, 1.40	—	—
0.15	—	—	1	0.81	—	—
0.17	1	1.23	—	—	—	—
0.20	2	0.71, 1.27	3	ND, 0.50, 0.68	—	—
0.33	—	—	1	ND	—	—
0.60	—	—	1	1.51	—	—
0.70	—	—	1	2.10	—	—
0.80	—	—	1	1.72	—	—
1.0	—	—	1	2.00	1	3.64
6.0	—	—	1	5.70	—	—
12.0	—	—	1	8.53	—	—

[a] % by weight.
[b] CE refers to Canada Eastern.
[c] ND = nondetectable.

trial, post-mortem examinations, which included weighing major organs, were carried out.

Poultry.—Experiments were conducted with 96 White Leghorn adult cockerels and 306 pullets. After a 24 h fast, cockerels were force-fed 50 g aliquots of cracked or cracked and pelleted clean wheat, vomitoxin-contaminated wheat, or a 50 + 50 (w/w) mixture. Starting 24 h after being force-fed, each bird was fed ad libitum for the next 7 days with the same wheat preparation, respectively. Individual body weights were obtained at the beginning and end of the ad libitum feeding period. Pullets were given wheat-soy (19) diets that contained either clean wheat, vomitoxin-contaminated wheat, or a 50 + 50 mixture for 10 weeks. Feed intake, body weight change, and egg production were recorded. Post-mortem examinations were then done on 6 cockerels for each wheat preparation and 18 pullets for each experimental diet.

The control sample, defined as clean winter wheat, contained no detectable levels of vomitoxin and only trace amounts (<0.01 ppm) of zearalenone.

Field Studies

When outbreaks of suspected mycotoxicoses in Ontario and Quebec were brought to our attention, on-site visits were carried out (by the authors E.R.F. and H.L.T.) to acquire information on the nature and extent of mycotoxin problems. Representative samples were collected for chemical analysis for mycotoxins.

Results and Discussion

Chemical Analysis

Although several surveys have been carried out for vomitoxin in corn and other field crops (11, 14, 15, 20, 21), this paper is the first reported survey of vomitoxin levels in Ontario white winter wheat. In the fall of 1980, a larger than normal number of winter wheat samples were submitted by local elevators in Ontario to the Canadian Grain Commission for official grading. This was due to the large quantity of wheat which was being down-graded to "Canada Feed" grade by the local elevator operators because of the high degree of sprouting found in the wheat. Many samples contained higher than normal amounts of pink-stained kernels. Forty-one samples were submitted to Agriculture Canada for chemical analysis, and many contained vomitoxin. The highest levels of vomitoxin contamination were found in wheat from the counties of Elgin, Kent, and Lampton located between Lake Huron and Lake Erie in southwestern Ontario. Twenty-eight samples were graded as "Canada Feed;" 9 were graded as No. 3, 4, or 5 Canada Eastern (CE) white winter wheat; and 4 had no official grade (Table 2). The percent *Fusarium*-stained kernels ranged from a low of 0.01% to a high of 12%.

No measurable amounts of vomitoxin were found in samples containing 0.10% or less of *Fusarium*-stained kernels. However, analysis did indicate much higher vomitoxin levels in Canada

Table 3. Analysis for vomitoxin in samples from the Ontario white winter wheat crop—boatload samples

Fusarium	Numerical grades (Nos. 2–5 CE)[b]		Canada Feed grade	
stained kernels %[a]	No. of samples	Vomitoxin, ppm	No. of samples	Vomitoxin, ppm
0.00	6	3-ND, 0.14, 0.16, 0.30	2	ND
0.01	—	—	2	ND
0.02	—	—	3	2-ND, 0.25
0.03	2	0.28, 0.41	2	ND, 0.38
0.04	—	—	2	0.15, 0.23
0.05	1	ND	2	ND, 0.25
0.06	—	—	2	ND
0.07	1	0.32	–	–
0.08	—	—	1	ND
0.10	1	0.16	5	ND, 0.06, 0.08, 0.36, 0.38
0.12	—	—	1	0.50
0.16	1	0.14	1	0.46
0.17	—	—	1	0.42

[a] % by weight.
[b] CE refers to Canada Eastern.

Feed grade wheat and the 4 samples of ungraded wheat than in the wheat which made numerical grades, i.e., grade Nos. 3-5 CE. Forty-three percent of the Canada Feed samples, 33% of the numerical grade samples, and 1 of 4 of the ungraded samples showed vomitoxin levels above the 0.05 ppm limit of detection.

Multi-mycotoxin analysis indicated that none of the mycotoxins screened for was present at levels above the limits of detection in any of the 41 samples analyzed.

Twenty-four samples representing boatloads or partial boatloads of blended wheat from shipments totaling 207 067 tons were Canada Feed grade. Table 3 shows the vomitoxin levels found in the 36 official samples drawn by the Canadian Grain Commission during boatloading operations. Compared with the Canada Feed grade samples from producers (Table 2), the average level of vomitoxin was much lower in the blended boatload samples (Table 3). For boatload samples, vomitoxin levels in the "Canada Feed" grade samples were essentially the same as in the numerical grades.

There were noticeable differences in the percentage of *Fusarium*-stained kernels. In the boatload samples of Canada Feed wheat (Table 3), 87% contained 0.10% or less of *Fusarium*-stained kernels compared with 43% in the individual producer's shipments (Table 2). When weighted for the size of shipments, samples containing 0.10% or less *Fusarium*-stained kernels accounted for 80% of the Canada Feed wheat shipped through the Great Lakes system. However, a considerable proportion of the boatload samples containing low levels of stained kernels showed measurable levels of vomitoxin, while none of the producers' shipments with 0.10% or less of *Fusarium*-stained kernels contained vomitoxin. A possible explanation is that blending resulted in dilution to the point that the *Fusarium*-staining could not be detected, yet chemical analysis could measure vomitoxin content.

Mycology

Neish and Cohen (22) analyzed vomitoxin-contaminated samples of white winter wheat from southwestern Ontario for the presence of *Fusarium* species. *Fusarium graminearum* Schwabe, *F. poae* (Peck) Wollenweber and *F. sporotrichioides* Sherbakoff sensu Seemüller were isolated. *F. graminearum* was the predominant species and, of 6 isolates tested, all produced vomitoxin in vitro. Canadian isolates of the other 2 species are not known to produce vomitoxin.

Feeding Studies – Swine

In 1973, Vesonder et al. (9) isolated an emetic factor, vomitoxin, from *Fusarium*-infected corn. Forsyth et al. (23) demonstrated a dose-response relationship between dietary vomitoxin levels (0–40 ppm) and feed consumption in swine. At 3.6 ppm vomitoxin, they reported a 20% decrease in feed intake.

Feeding trials were carried out in our animal facilities to assess the nutritional/toxicological effects of low levels of vomitoxin-contaminated wheat on swine. When pigs were fed grower-

Table 4. Field studies of suspected mycotoxin outbreaks in Ontario and Quebec

Location	Species	Symptoms	Vomitoxin, ppm	Zearalenone, ppm
Smithville, Ontario	pig	vomiting; feed refusal	0.95	0.13
Barrie, Ontario	pig	pink mold; feed refusal	0.03	0.007
Buckingham, Quebec	pig	vomiting; feed refusal; 100 out of 500 pigs died	0.14	<0.002
Buckingham, Quebec	cattle	2 out of 7 cattle died		
Hanover, Ontario	pig	vomiting	<0.05	<0.2

finisher diets containing contaminated wheat (1.0 ppm vomitoxin), feed refusal in some pens of pigs was evident during the first 1–3 days. Average daily gains for the 21-day study were certainly related to vomitoxin levels: 0.68 kg/day for pigs fed vomitoxin-contaminated wheat vs 0.82 kg/day for pigs fed a diet containing contaminated wheat diluted 50 + 50 with clean wheat. In contrast, pigs fed the control diet gained 0.95 kg/day. Post-mortem examinations of the pigs at the end of the study did not identify any organ damage or differences in organ weights corrected for body weights. There was no evidence that the vomitoxin-contaminated feed caused vomiting in any of the treated animals.

Feeding Trials – Poultry

Preliminary studies by Featherston (24) suggested that poultry may be more tolerant; no differences were observed in weight gains and feed efficiencies of chicks fed normal and *Gibberella zeae*-infected corn. Adams and Tuite (25) reported decreased egg production and feed consumption of laying hens fed corn heavily damaged by *G. zeae;* no analyses were carried out to determine what toxin levels were fed. The decreased performance could have been due to vomitoxin and/or other contaminants in the feed. More recently, Huff et al. (26) reported an acute LD_{50} for vomitoxin of approximately 140 mg/kg for young broiler chickens.

The feeding trials in our laboratory were designed to investigate nutritional and toxicology parameters at much lower levels of vomitoxin administered daily over a period of several weeks. Close observation of adult roosters immediately after they were force-fed 50 g aliquots of clean wheat, vomitoxin-contaminated wheat, or a 50 + 50 mixture of these wheats, or when they received these wheat preparations ad libitum indicated no noticeable changes in their behavioral patterns and appearance. Providing the preparations in the form of either cracked or cold pelleted wheat had no significant effect on the results. Post-mortem examinations of the roosters at the completion of the 7-day experiment indicated no gross lesions in the internal organs.

Incorporating vomitoxin-contaminated wheat into laying diets for young pullets indicated that up to 0.7 ppm vomitoxin in the diet had no effect ($P > 0.05$) on feed intake and egg production. There was no indication that ingestion of the vomitoxin-contaminated wheat produced any organ damage (27).

A great deal of research is still needed to establish the levels of vomitoxin-contaminated grain which are safe to feed to various classes of farm animals. Many questions need to be answered, for example, are the symptoms observed due to vomitoxin or to interactions of several toxins present in the feedstuff? Is there a response to the fungus itself? Is there a purely nutritional implication? Several studies have not reported vomitoxin levels for the feeds used. Our findings indicate that feed consumption and weight gains can be reduced in swine fed vomitoxin-contaminated wheat at levels as low as 0.3 ppm. In the poultry experiments, no major differences were observed in feed consumption or egg production. However, no gross organ damage was observed in any of the pigs or poultry examined at the termination of experiments.

Field Studies

The results of 4 field cases during the summer–autumn of 1980 are summarized in Table 4; in all reports, pigs were affected. In 2 instances, commercial pig grower diets were suspect, while in the others, barley and wheat screenings were implicated. Based on our recent work with pigs, vomitoxin might have been an important contributing factor in the Smithville, Ontario, pigs. In the other cases, the levels of vomitoxin and zearalenone were lower than levels expected to produce typical reactions.

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Preparation and Characterization of Acid Dehydration Products of Aflatoxicol

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Treatment of aflatoxicol with acid resulted in the formation of dehydrated derivatives with the 2,3 double bond either hydrated or unaffected. Chromatographic and physicochemical analyses of the purified derivatives revealed that hydration of the 2,3 double bond of aflatoxicol with simultaneous dehydration occurred when sulfuric acid was used. When a weaker acid such as chloroacetic acid was used, no hydration occurred, and 2 isomeric forms of the dehydrated aflatoxicol were obtained. Chicken embryo assay and the Ames test revealed that dehydrated aflatoxicols were much less toxic and mutagenic than aflatoxicol. A mechanism for the dehydration of aflatoxicol is proposed.

Aflatoxin B_1 is produced by some toxic isolates of *Aspergillus flavus* and *A. parasiticus*, and has been found to be one of the most potent naturally occurring carcinogens. Because of the widespread nature of the fungi in the environment and the reported natural occurrence of aflatoxin B_1 in many commodities, its presence in foods and feeds has become a potential hazard to human and animal health (1, 2). Consequently, efforts have been made to develop a rapid and sensitive method for monitoring this toxin in foods and feeds (3). During the past few years, we have been involved in the development of immunoassays for a number of mycotoxins including aflatoxin B_1 and have shown that this method is one of the most promising techniques for aflatoxin analysis (4–8).

Whereas B_1 is primarily found in agricultural commodities, this toxin is known to be metabolized by animals and humans to a number of hydroxylated derivatives (9, 10). Thus, it is likely that B_1 exists as its metabolites in animal tissues and biological fluids. Among many B_1 metabolites, aflatoxin M_1 was the predominant metabolite in milk (11). Aflatoxicol has been considered to be one of the most important B_1 metabolites because there is a correlation between the presence of this metabolite in animal tissues and body fluids with the toxicity of B_1 in different animal species (10, 12, 13). Since we have already developed an enzyme-linked immunoassay for aflatoxin M_1 (14), we attempted to elicit specific antibodies against aflatoxicol. In the study of different methods for preparing aflatoxicol derivatives to be used for the subsequent coupling reaction, we found that aflatoxicol converted to dehydrated derivatives after acid treatment. In this paper, we describe conditions leading to the formation of such derivatives, and the chemical, physical, and biological properties of the acid dehydration product. A possible mechanism for formation of such derivatives is proposed.

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Experimental

Materials

Aflatoxin B_1 and aflatoxicol were prepared according to the methods of Chu (15) and Hsia and Chu (16), respectively. Chloroacetic acid was obtained from Eastman Kodak Co. (Rochester, NY). d_6-Dimethyl sulfoxide (d_6-DMSO) was obtained from Aldrich Chemical Co. (Milwaukee, WI) and silica gel 60 (70–230 mesh) was purchased from MC/B Manufacturing Chemists, Inc. (Cincinnati, OH). All other chemicals and organic solvents were analytical reagent grade.

Preparation of 2-Hydroxyl-Dehydrated Aflatoxicol (R_{2x})

A mixture of 3.5 mg aflatoxicol and 50 μL 10% H_2SO_4 in 3.5 mL acetone was refluxed 2 h. Thin layer chromatographic (TLC) analysis revealed that all aflatoxicol converted to a compound that had a lower R_f value. The mixture was cooled to room temperature, and the solvent was removed by evaporation. The residue was dissolved in 50 mL chloroform and washed with three 10 mL portions of water. The chloroform layer was dried over anhydrous Na_2SO_4 and concentrated to a small volume. The concentrated solution was loaded onto a silica gel 60 column (1.5 × 15 cm), washed with 200 mL 3% acetone in chloroform, and eluted with 5% acetone in chloroform. Total amount of pure 2-hydroxyl-dehydrated aflatoxicol eluted was 42 absorbance units at 350 nm.

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Preparation of Dehydrated Aflatoxicol (R_x)

A mixture of 10 mg aflatoxicol and 250 mg chloroacetic acid in 10 mL acetonitrile was refluxed 2 h. TLC analysis revealed that all aflatoxicol was converted to a compound that had a higher R_f value. The mixture was cooled to room temperature, and the solvent was removed by evaporation. The residue was dissolved in 50 mL chloroform and washed with three 10 mL portions of water. The chloroform layer was dried over anhydrous Na_2SO_4 and concentrated to a small volume. The concentrated solution was chromatographed on a Florisil column (2.5 × 25 cm) equilibrated with methylene chloride. The column was washed with 20 mL methylene chloride. The dehydrated aflatoxicol (R_x) was eluted from the column with 600 mL 2% acetone in methylene chloride. The fractions containing the eluted R_x were combined and concentrated to yield 150 absorbance units (A_{352} nm) of pure and 63 absorbance units (A_{352} nm) of impure dehydrated AFL. About 90 absorbance units (A_{352} nm) of an unknown impurity was obtained by washing the column with 20% methanol in methylene chloride.

Analysis

Ultraviolet (UV) spectra were determined in a Beckman Model 25 spectrophotometer with a 1 cm light path. Mass spectra were obtained on a Hewlett-Packard Model 5980A mass spectrometer (Palo Alto, CA). Proton nuclear magnetic resonance spectra (NMR) were measured in a Bruker Model HX-90E spectrometer (Bruker Scientific, Inc., Elmsford, NY). High performance liquid chromatographic (HPLC) separations were performed on a Waters Model ALC 202 liquid chromatograph equipped with an A6000 pump, U6K septumless injector (Waters Associates, Milford, MA) and a variable wavelength UV detector set at 350 nm (Laboratory Data Control, Inc., Riviera Beach, FL). A μBondapak C_{18} column (4 mm × 30 cm, 10 μm particle size; Waters Associates, Inc., Milford, MA) was used for all separations. Precoated plastic TLC sheets (0.2 mm of silica gel G60, MC/B Manufacturing Chemists, Inc.) were developed with appropriate solvent systems. After development, plates were visualized under UV light at long wavelength.

Chicken Embryo Assay

The toxicity of the dehydrated aflatoxicol was tested on 7-day-old chicken embryos (Leghorn-New Hampshire cross, supplied by the Department of Poultry Science, The University of Wisconsin, Madison) according to the method described previously (17).

Mutagenic Assay

Bacterial mutagenesis of the dehydrated aflatoxicol was assayed according to the agar overlay method of Ames et al. (18) as modified by Drinkwater et al. (19) using *Salmonella typhimurium* TA98.

Results and Discussion

Characterization of 2-Hydroxyl Dehydro-Aflatoxicol

Because the initial objective of the present study was to prepare an aflatoxicol derivative for subsequent conjugation to a protein, our first approach was to prepare the hemisuccinate. When we found that the hemisuccinate was very unstable, we used another approach by preparing a derivative through the hydration of the 2,3 double bond of aflatoxicol. However, extensive analyses of the hydration product prepared by treatment of aflatoxicol in acetone with sulfuric acid revealed that the new derivative was not the 2-hydroxyl derivative. TLC analysis showed a diffused spot with an R_f value of 0.2 when developed in acetone–chloroform (1 + 9, v/v). HPLC analysis revealed 2 distinct peaks with retention times of 4.5 min (70%) and 5.5 min (30%) after elution with acetonitrile–water–acetic acid (35 + 65 + 1, v/v/v) at a flow rate of 2 mL/min. Although those properties suggested that hydration might occur, mass spectral data showed that the parent peak was at m/z 314 (39%), identical with the molecular weight of the original aflatoxicol. Other mass spectral peaks including m/z (relative intensity) at 296(36), 286(21), 285(100), 259(16), and 257(55) were observed. Because the M − 18, M − 29, and M − 57 peaks are similar to the fragmentation pattern of B_{2a} (20, 21), present results suggest that hydration at the 2,3 position had indeed taken place. The UV spectrum of the new derivative in methanol showed maximal absorptions at 350 and 258 nm, compared with aflatoxicol which absorbs maximally at 330 nm. The 20 nm bathochromic shift suggests that an additional conjugation double bond is formed. From the results, a structure for the new derivative, R_{2x}, is proposed in Figure 1. The acid hydration at the 2,3 double bond position of aflatoxicol must occur accompanying an acid dehydration of the hydroxyl group in the molecule to give a compound (R_{2x} of Figure 1) with molecular weight identical to the parent.

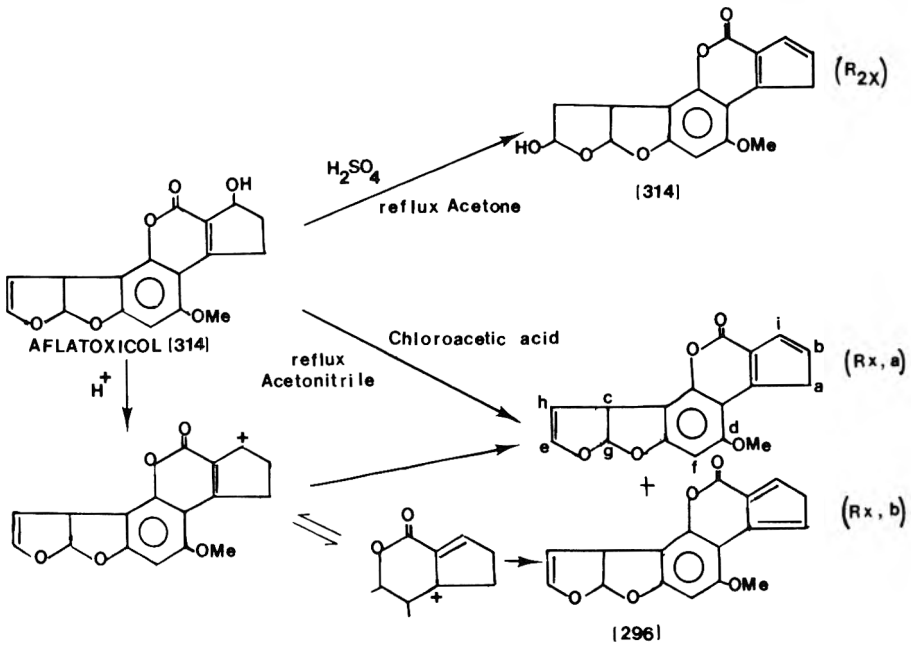

Figure 1. Preparation of 2-hydroxyl dehydrated aflatoxicol and dehydrated aflatoxicol.

The 2 peaks observed in the HPLC analysis might be the 2 isomers which will be discussed later.

Characterization of Dehydro-Aflatoxicol (R_x)

In view of the formation of dehydrated product after treatment of aflatoxicol in acid, we attempted to prevent dehydration by lowering the reaction temperature, using less acid catalyst, and using a weaker acid catalyst. However, we could not obtain a hydrated product without dehydrating the hydroxyl group. When a weaker acid catalyst such as chloroacetic acid was used, the dehydrated product could be obtained in pure form. No hydration of the 2,3 double bond was observed.

TLC analysis (acetone-chloroform; 1 + 9; v/v) of the compound showed 2 distinct spots with R_f = 0.79 (30%) and 0.71 (70%). HPLC analysis with acetonitrile–water–acetic acid (50 + 50 + 1, v/v/v) at a flow rate of 2 mL/min also gave 2 peaks with retention times of 4.7 min (70%) and 5.7 min (30%). The compounds absorbed maximally at 352 and 258 nm in methanol. Mass spectral analysis revealed peaks at m/z (relative intensity) 297(20), 296(100), 268(24), 267(67), 253(27), and 225(32). Assignment for the protons of the NMR(d_6-DMSO), as designated to the structure of R_{xa} (Figure 1) for ppm downfield from tetramethyl silane (abbreviations: s-singlet, d-doublet, t-triplet, m-multiplet, b-broad) are: δ3.50(2H, s, H_a), δ3.94(3H, s, H_d), δ4.81(1H, d of t, H_c), δ5.42(1H, t, H_h), δ6.69(2H, b-s, H_e and H_f), δ6.92(1H, d, H_g), δ7.14(1H, d, H_b), and δ7.44(1H, d, H_i).

The physicochemical properties of the dehydrated aflatoxicol are similar to the 2-OH-dehydrated compound in several respects. Both derivatives had 2 isomers and both also had similar UV spectra. The presence of 2 isomeric forms of both dehydrated products is likely due to the formation of carbonium ion which undergoes rearrangement to a more stable form. The mechanism for the dehydration of aflatoxicol is shown in Figure 1. The molecular weight (m/z 296) and the NMR spectrum of the compound agree very well with the proposed structure of dehydrated aflatoxicol. Structure R_{xa} in Figure 1 is the major product, and is probably more stable than R_{xb}. The NMR coupling patterns of protons a, b, and i are of particular interest. Proton a is expected to couple with proton b to give a doublet. However, due to the planar structure of the cyclopentadiene ring and the dihedral angle between protons a and b, the coupling constant becomes small; thus, a singlet was observed for proton a. For the same reason, proton b does not couple with proton a; thus it is only split by proton i to give a doublet.

Biological Activity of Dehydrated Aflatoxicol

Since there is considerable evidence indicating that the 2,3 double bond in aflatoxicol is important for its biological activity (22), we studied the effect of the additional bond in the dehydrated aflatoxicol molecule on its biological activity. Results of the chicken embryo test revealed that the dehydrated compound was less toxic than aflatoxicol to 7-day-old chicken embryos. All 9 embryos in the aflatoxicol group (2 μg/egg) failed to hatch at the end of the incubation period, whereas only half of the embryos injected with high concentrations of dehydrated aflatoxicol (10 μg/egg) failed to hatch. At lower concentrations, the dehydrated aflatoxicol showed no toxic effect on the chicken embryos.

Mutagenesis assays showed that the dehydrated aflatoxicol was also less mutagenic than aflatoxicol, which is known to be less mutagenic than aflatoxin B_1 (23). Aflatoxicol produced approximately 700 revertant colonies at a level of 50 ng/plate, whereas the dehydrated compound produced only 80 revertant colonies at the levels between 50 and 500 ng/plate tested.

Previous studies have shown that although the 2,3 double bond in the aflatoxin B_1 molecule is essential for biological activity, the cyclopentenone ring part also plays an important role (22). It has been suggested that whereas the 2,3 double bond in B_1 is directly involved in the covalent binding to the macromolecules (24–27), the extensive conjugated double bonds may be important in the noncovalent interaction with the substrate, thus enhancing the covalent bindings (22, 28). In the present study, we found that the toxicity and mutagenicity of R_x, in which the carbonyl group of aflatoxin B_1 was replaced by an endocyclic double bond, were considerably lower than for aflatoxicol. This replacement to give a cyclopentadienyl ring should confer greater rigidity to the molecule, which may affect subsequent metabolism and macromolecule bindings. Although dehydrated aflatoxicol can be prepared very easily by chemical methods, the question of whether it is produced in vivo by animals as one of the B_1 metabolites warrants further study.

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High Pressure Liquid Chromatographic Determination of Zearalenone in Chicken Tissues

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A method is reported for the extraction and analysis of zearalenone in chicken fat, heart muscle, and kidney tissue by using high pressure liquid chromatography (HPLC). Zearalenone is extracted with acetonitrile, cleaned up with hexane, and extracted further with ethyl acetate. Zearalenone is determined by HPLC using a reverse phase radial compression separation system, an ultraviolet absorbance detector, and a mobile phase of acetonitrile-water (60 + 40) (v/v). Recoveries of zearalenone added at levels from 50 to 200 ng/g are in the range 82.6-95.1%.

Zearalenone is an estrogenic mycotoxin produced by *Fusarium roseum* (*F. graminearum*) and other *Fusarium* spp. and commonly occurs in grains and commercial preparations of mixed feeds (1-5). The importance of this mycotoxin and its chemistry, biological activity, and occurrence in food have been reported (6-10).

Corn and other grains are used extensively in poultry feed, so the likelihood that chickens consume zearalenone-contaminated feed is certainly very real. Recent studies have shown that chickens are not very susceptible to the effects of zearalenone (11-13). However, residues of zearalenone in chicken tissues may be of concern in terms of food safety. It has been shown that farm animals exposed to zearalenone in their feed can transmit residues of the mycotoxin in their milk (14, 15).

Because zearalenone might enter the human food chain via chicken tissues, precise analytical methods are required to determine levels of zearalenone in poultry. Although numerous methods are available for determination of zearalenone in plant material, the procedures are not generally applicable for analysis of animal tissues (16, 17). This paper describes a simple, rapid, and sensitive HPLC method for the quantitative determination of zearalenone in chicken fat, heart muscle, and kidney tissue. The method involves a solvent extraction step, sample cleanup by liquid-liquid partition, final extraction, and zearalenone determination by reverse phase HPLC with ultraviolet absorbance detection.

METHOD

Apparatus

(a) *High speed homogenizer.*—Polytron (Brinkmann Instruments, Westbury, NY).

(b) *Centrifuge.*—International centrifuge Model UV (International Equipment Co., Needham, MA).

(c) *Evaporators.*—(1) Buchi Rotavapor-R (Rinco Instruments Inc., Greenville, IL). (2) N-Evap Analytical Evaporator Model 112 (Organomation Associates Inc., Northborough, MA).

(d) *High pressure liquid chromatograph.*—Waters Model ALC-204 equipped with M6000 pump, U6K septumless injector, and Model 440 UV detector set at maximum sensitivity of 0.005 absorbance unit full scale (AUFS) and with 280 nm primary filter (Waters Associates, Inc., Milford, MA).

(e) *Variable wavelength UV detector.*—Tracor 970A variable wavelength detector set at sensitivity of 0.01 AUFS (Tracor Inc., Austin, TX).

(f) *Liquid chromatographic column.*—Waters Radial-PAK A liquid chromatography cartridge (reverse phase). Operating conditions: chart speed 0.5 in./min; flow rate 1.5 mL/min; temperature ambient; injection volume 10 μL.

(g) *HPLC recorder.*—Series B 5000 Omniscribe (Houston Instrument, Austin, TX).

(h) *Integrator.*—Model 3390A reporting integrator (Hewlett-Packard, Avondale, PA).

Reagents

(a) *Solvents.*—All solvents were distilled in glass (Burdick & Jackson, Muskegon, MI).

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

(c) *Zearalenone standard solution.*—Dissolve 1.0 mg zearalenone in 1 mL acetonitrile. (Zearalenone was a gift from M. C. Backman, IMC Corp., Terre Haute, IN.)

(d) *Chicken tissues.*—Obtain abdominal fat, heart muscle, and kidney tissue from 16-week-old leghorn pullets (ca 1.2 kg body weight) fed

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a zearalenone-free diet for 2 weeks before euthanasia with CO_2.

Extraction

Weigh 4 g samples of fat, heart muscle, and kidney tissue in duplicate and cut into small pieces. Add 8 mL water to each sample and homogenize in 50 mL glass-stopper centrifuge tubes. Spike duplicate tissue homogenates with zearalenone at the following levels: 50, 100, 150, and 200 ng/g. Use a tissue sample with no zearalenone added as a control in each case. Let stand 1 h. Add 25 mL acetonitrile, mix, and let stand 1 h, mixing frequently. Centrifuge 15 min at 2500 rpm. Add 2 mL saturated lead acetate solution, mix gently, and centrifuge 15 min at 2500 rpm.

Cleanup

Decant supernate into 125 mL separatory funnel. Add 10 mL hexane and collect acetonitrile–water fraction in another separatory funnel. Add 25 mL ethyl acetate. Discard water fraction. Collect acetonitrile–ethyl acetate fraction in 100 mL round-bottom flask and evaporate by using rotary evaporator. Remove residual water by azeotropic mixing with acetonitrile. Dissolve residue in acetone and transfer to test tube. Evaporate to dryness under gentle stream of nitrogen in fume hood.

Liquid Chromatography

Prepare serial dilutions of standard zearalenone solution and make triplicate injections of 50 and 100 ng zearalenone into liquid chromatograph. Determine mean peak areas for 50 and 100 ng zearalenone. Add 500–750 μL acetonitrile to sample extracts. Inject, in triplicate, 10 μL from each duplicate tissue sample. Determine amount of zearalenone in samples by comparing mean peak areas with peak areas obtained from known concentrations of standard zearalenone.

To confirm detection of zearalenone, make sequential 10 μL injections of 50 ng standard zearalenone and tissue extracts with variable wavelength absorbance detector set at consecutive wavelengths of 236 and 274 nm. Record peak areas and, in each case, determine peak area ratio 236/274. Peak area ratios obtained for tissue extracts should agree within 10% of that obtained for standard zearalenone (4, 17–19).

Results and Discussion

Table 1 gives the percentage recovery of zearalenone from spiked chicken tissues after the described extraction and cleanup, as measured by HPLC. The mean recoveries of zearalenone added at levels from 50 to 200 ng/g are in the range 82.6–95.1%.

Table 1. Percentage recovery [a] of zearalenone added to chicken tissues

Added, ng/mL	Kidney	Heart	Fat
50	84.4 ± 3.2	82.6 ± 3.6	88.4 ± 3.9
100	87.4 ± 2.7	84.9 ± 4.6	89.9 ± 3.7
150	92.1 ± 2.3	94.4 ± 3.0	94.9 ± 3.1
200	92.9 ± 1.9	92.6 ± 4.0	95.1 ± 3.1

[a] Each value represents the mean ±SD for 6 analyses.

Under the HPLC conditions described, the analysis of chicken fat, heart, and kidney tissue extracts yielded well resolved peaks (Figure 1). No problems were encountered in detecting and quantitating zearalenone. The retention times and peak areas were highly reproducible for the range of zearalenone concentrations used in this study. The relative standard deviations were less than ±4% for triplicate injections from duplicate samples for each level of zearalenone added. The mean retention time for zearalenone was 5.0 min.

Figure 1 illustrates a typical chromatogram of 50 ng zearalenone standard and of a chicken fat sample to which zearalenone was added at the 150 ng/g level. The peak represents 5.5 ng zearalenone (91.6% recovery) obtained from a 10 μL injection of fat extract. The zearalenone peak is unaffected by any interfering peaks. Chromatograms obtained from heart and kidney tissue extracts also gave well separated zearalenone peaks. A standard curve for zearalenone gave a linear response from 1 to 50 ng. Under the chromatographic conditions described, as little as 1 ng zearalenone standard can be detected (data not shown). It is quite possible that this method will allow detection of zearalenone in these tissues at much lower levels than those included in this study. Preliminary data obtained in this laboratory indicate that the sensitivity for fat samples is 6.8 ng zearalenone/g tissue. It also may be possible to detect lower levels by increasing the initial tissue sample size and/or increasing the HPLC injection volume from 10 to 20 μL. Further work is in progress to investigate these possibilities.

Various analytical procedures have been developed for detecting zearalenone, many of which are difficult and laborious. The analysis of chicken tissues by HPLC, using a radial compression separation system, for the presence of

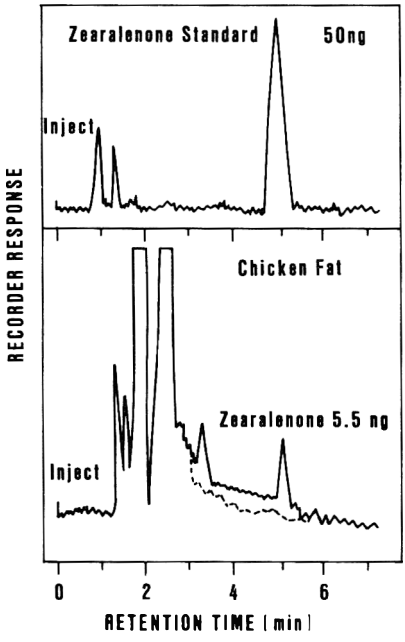

Figure 1. Chromatogram (schematic) of zearalenone standard (50.0 ng) and chicken fat extract with 150 ng/g added zearalenone (91.6% recovery). Dotted lines refer to control fat sample. See text for HPLC conditions.

zearalenone has not been previously reported. The HPLC procedure described here is simple, rapid, and sensitive. The extraction and cleanup procedure has been designed to minimize the loss of toxin during extraction. It was also an effective method for removing interfering substances in the chicken tissues. This technique was not satisfactory for the analysis of skeletal muscle. In some muscle tissue samples, the resolution of zearalenone was confounded by the interference of an unidentified peak. Although not the subject of this paper, the technique described may prove useful for monitoring zearalenone in the tissues of chickens and possibly other food animals suspected of ingesting contaminated feeds.

Confirmation techniques available for zearalenone include the collection of the zearalenone-contained eluate fraction from the liquid chromatograph and the analysis of the material by mass spectrometry; the formation of derivatives and their subsequent analysis; and the use of TLC with various spray reagents. The confirmation procedure employed was based on the absorbance ratioing technique described by other workers, and was simple, practical, and satisfactory (4, 17–19).

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PESTICIDE RESIDUES

Colorimetric Determination of Propoxur and Its Residues in Vegetables

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A method has been developed for the determination of propoxur (*o*-isopropoxyphenyl *N*-methylcarbamate) based on the hydrolysis of propoxur with methanolic potassium hydroxide to its phenol and coupling with diazotized 4,4-diaminodiphenyl sulfone. The orange complex formed has an absorption maximum at 500 nm and obeys Beer's law in the range 0.25-5.0 μg/mL. The method can be applied to levels as low as 0.5 ppm propoxur from vegetables.

Propoxur (*o*-isopropoxyphenyl *N*-methylcarbamate) is a nonsystemic carbamate insecticide used on a large scale against a broad spectrum of insects in field crops, fruits, vegetables, ornamentals, and flowers. It has also been extensively used for residual indoor applications against mosquitos, cockroaches, and flies. Propoxur residues have been determined by gas-liquid chromatographic (GLC) methods (1-6), colorimetric methods (7-13), thin layer chromatographic (TLC) methods (14, 15), and TLC-enzymatic methods (16, 17). Although GLC is ideal for detecting and estimating propoxur, its use is often limited by availability and cost of equipment. Unavailability of other chromogenic reagents has, at times, necessitated a search for an alternative chromogen.

We developed a sensitive colorimetric method, using the chromogenic salt 4,4-diaminodiphenyl sulfone, which is more sensitive than other colorimetric methods (7-13).

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Perkin Elmer Model 475 with 1 cm silica cells.

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

(c) *Chromatographic column.*—400 × 20 mm id Pyrex glass column with integral reservoir.

(d) *Propoxur.*—99% pure (Bayer (India) Ltd, Thana, Maharashtra, India). Prepare working standard containing 100 μg/mL methanol.

(e) *Methanolic KOH.*—Prepare 1N KOH in methanol.

(f) *Sodium nitrite.*—AR grade (Sarabhai Merc Ltd, Baroda, India). Prepare 1% solution in water.

(g) *4,4-Diaminodiphenyl sulfone.*—Burroughs Wellcome & Co. (India) Private Ltd, Bombay-400023, India). Prepare 0.5% solution in methanol.

(h) *Phosphate buffer.*—Prepare pH 5 buffer by adding 15 mL of 2/15M $NaHPO_4.7H_2O$ to 1 L 2/15M KH_2PO_4.

(i) *Florisil.*—60-100 AR grade (available from Fluka, Switzerland). Heat 24 h at 130°C to remove moisture. Deactivate by adding 2.5% water (2.5 mL water + 97.5 g dried Florisil) and let equilibrate 24 h in tightly stoppered bottle before use.

Preparation of Standard Curve

Pipet ca 0.5 mL portions of 4,4-diaminodiphenyl sulfone to clean, dry test tubes. Diazotize by adding 1 mL 1% $NaNO_2$ and 1 mL 1N HCl to each test tube.

Add 0.0, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.0 mL aliquots of propoxur working standard solution (5-100 μg) into separate test tubes. Add 2 mL 5% methanolic KOH and 2 mL water to each tube. Shake well and keep tubes on boiling water bath 30 min. Remove tubes and let stand 10 min at room temperature. Quantitatively transfer diazotized reagent into tubes containing hydrolyzed propoxur. An orange dye with an absorption maximum at 500 nm will form immediately (color is stable 12 h). Let stand 5 min for full color development. Quantitatively transfer colored solution to 20 mL volumetric flask and dilute to volume with methanol. Read absorbance of orange dye at 500 nm in spectrophotometer against blank similarly prepared. Plot of concentration of propoxur (0.25-5.0 μg/mL) against absorbance will yield straight line.

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Determination of Recovery

Sample extraction.—Extract propoxur residue in vegetables by cleanup procedure of Stanley et al. (3): Weigh 50 g sample (okra, cabbage, or beans) into blender jar. Spike samples with 25–250 μg propoxur in 1 mL $CHCl_3$. Add 5 mL water and 200 mL acetone and blend 5 min at high speed. Filter sample through 12.5 cm Whatman No. 42 paper covered with ca 6 cm Hyflo Super-Cel in Buchner funnel. Return filter cake to blender and add 200 mL $CHCl_3$. Blend sample at high speed for 3 min and filter again. Wash blender jar and filter cake with 100 mL acetone and 100 mL $CHCl_3$. Evaporate filtrate to dryness on rotary vacuum evaporator in 38°C water bath. Transfer the residue in flask to 250 mL separatory funnel with 50 mL $CHCl_3$ and 25 mL pH 5 phosphate buffer. Shake separatory funnel 30 s and let stand 5 min. Drain $CHCl_3$ layer into 250 mL Erlenmeyer flask and extract buffer solution twice more with 50 mL portions of $CHCl_3$. Combine all $CHCl_3$ phases in flask. Evaporate $CHCl_3$ extract to dryness on rotary vacuum evaporator. Transfer residue in flask to 500 mL separatory funnel, using 150 mL hexane and 40 mL acetonitrile successively. Shake separatory funnel 30 s and drain acetonitrile layer into another separatory funnel containing 80 mL hexane. Shake second separatory funnel 30 s and drain acetonitrile layer into round-bottom flask. Add 40 mL fresh acetonitrile to first separatory funnel and repeat 2-stage extraction as described above. Evaporate combined acetonitrile phases to dryness and preserve residue for column chromatography as described below.

Column chromatography.—Tamp glass wool plug into bottom of chromatography tube with integral reservoir of $CHCl_3$. Slowly sprinkle in 15 g Florisil and let settle. Top column with anhydrous sodium sulfate up to 2 cm and drain solvent to top of column. Transfer residue in flask to column with 4 washes of 5–10 mL $CHCl_3$. Elute column with total volume of 250 mL $CHCl_3$, including washes, at 2–4 drops/s. Transfer $CHCl_3$ eluate to rotary vacuum evaporator and evaporate just to dryness. Dissolve residue in appropriate quantity of methanol and estimate amount recovered by method described under standard curve preparation. Use separate crop control (blank) for each vegetable.

Results and Discussion

To check the recovery of propoxur by the method described, vegetable samples (okra, cabbage, and beans) were spiked with known amounts of propoxur and analyzed. Recoveries varied from 90.1 to 96.3% (Table 1). The method can be used to determine levels as low as 0.5 ppm in a 50 g sample. Interference by other phenolic substances is eliminated by measuring the absorbance against a corresponding crop control (blank).

Table 1. Recovery (%) of propoxur from spiked vegetable samples (50 g sample)

Sample	Added, μg	Found, μg[a]	Rec., %
Okra	25	23.25 ± 0.00	93.0
	50	46.05 ± 0.21	92.1
	75	68.25 ± 1.45	91.0
	100	90.10 ± 0.40	90.1
Cabbage	25	24.07 ± 1.90	96.3
	50	47.00 ± 0.00	94.0
	75	69.90 ± 0.72	93.2
	100	91.00 ± 0.51	91.0
Beans	25	24.00 ± 0.35	96.0
	50	47.55 ± 1.50	95.1
	75	70.27 ± 0.70	93.7
	100	91.00 ± 1.20	91.4

[a] Average ± standard deviation of 5 analyses.

The linear relationship between the absorbance at 500 nm and the concentration of propoxur is valid up to 5 μg/mL reaction mixture. Beer's law is obeyed in the range 0.25–5.0 μg/mL.

Hydrolysis of propoxur in pure standard solutions and also from vegetable samples to its corresponding phenol is complete only if carried out on a boiling water bath for 30 min. When hydrolysis is performed for less than 30 min, transformation of propoxur to its corresponding phenol is incomplete. However, loss of propoxur occurs if hydrolyzed beyond this time limit.

The proposed colorimetric method is slightly more sensitive than the other colorimetric methods with reported sensitivities of 0.5–10 μg (7–13). Although the present method is not as sensitive as the gas–liquid chromatographic methods (1–6) and TLC–enzymatic methods (16, 17), the method is very useful for determining propoxur residue in vegetables (permissible limit 3.0 ppm).

Acknowledgment

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Colorimetric Method for Field-Screening Above-Tolerance Parathion Residues on and in Citrus Fruits

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A colorimetric technique has been developed which is suitable for use as a field-screening method for detecting above-tolerance levels of parathion on and in citrus fruits. By using this method, a grower should be able to postpone harvesting a crop until parathion residues are below tolerance level, so that the crop is safe to market. The method depends on the reaction of parathion with 4-(*p*-nitrobenzyl)-pyridine. Parathion is extracted by mixing chopped citrus rind with acetone in a hand-operated homogenizer. The extract is partially cleaned by a partitioning step before final cleanup with a Sep-Pak Florisil cartridge. The colored reaction solution is read at 560 nm by using a portable, rechargeable spectrophotometer. A single test can be completed in about 75 min; the average time per test when several are conducted sequentially is considerably shorter. The analytical system responds readily to <5 ppm parathion on or in 1 g navel orange rind, which corresponds to <1 ppm in the whole fruit. The present U.S. tolerance for parathion on or in citrus is 1 ppm on a whole fruit basis. Preliminary work indicates that the method should also be suitable for apples.

Earlier work in this department resulted in reports on a field method for estimating organophosphorus insecticide residues on citrus foliage (1, 2). We have modified and extended that work to enable field-screening for above-tolerance parathion residues on citrus. Development of a field method specifically for parathion on and in citrus is desirable because citrus is a major agricultural crop in California, and parathion is widely used on citrus. If accepted, the method would be used to help ensure a grower that his fruit would be in compliance with regulations. Thus, the grower could delay harvest until results indicated that residues had dissipated to a safe (tolerance) level for marketing.

Specificity is provided primarily in the main part of the cleanup technique, which uses Sep-Pak Florisil cartridges. Although absolute specificity is not provided, parathion is the most commonly used organophosphorus pesticide in California citriculture which would elute from Florisil under the conditions used, and in the fraction specified in the method.

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Experimental

Materials

Essentially the same as in Ref. 2 except a crystalline table salt bath on a 150°C hot plate was used. In the field, a campstove and related equipment as specified by Berck et al. (2) may be used. Caution: Campstoves can be hazardous in use.

Additional Equipment

(a) *Hand-operated homogenizer.*—15 mL (Cat. No. 37236, Bel-Art Products, Pequannock, NJ 07440), modified by making the Telfon® "pestle" very rough by cutting small pieces away from the entire surface with a razor blade.

(b) *Test tubes.*—15 × 150 mm, with Teflon-lined screw caps.

(c) *Cleanup cartridges.*—Sep-Pak® Florisil® cartridges (Waters Associates, Milford, MA 01757).

(d) *Microcells.*—For use in rechargeable, portable Bausch and Lomb mini Spectronic 20 spectrophotometer.

(e) *Disposable dropping pipets.*

(f) *Set of hand cork borers.*

Sample Preparation

Single fruit.—Peel fruit and select uniformly thick section of rind near blossom end. This represents portion with highest concentration of pesticide; analysis from this region thus adds a safety factor to final result. To further ensure examining maximum pesticide load, use fruit sampled from tree at 4-ft level, and from sprayer-row sides of tree (2). Measure rind thickness, and, from 5-mm thick piece of navel orange rind, cut disk by using No. 14 hand-operated cork borer. Average weight of 21–22 mm diameter disk will be about 1 g. (Individual operators will want to do their own calibration by determining average weight of several disks obtained from different uniform thicknesses by using cork borers of different sizes. Then use cork borer rind thickness combination that will give about 1 g sample.) With long-blade knife, cut disk into small 4–6 mm cubes on hard surface of masonite board, and then transfer entire

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sample into tube of hand-operated homogenizer. Although its use is tedious, this apparatus permits homogenization of chopped sample without use of motorized power (see below).

Representative whole fruit sample.—Cut disk from rind sections from each of at least 20 fruits. Cut all disks into cubes, as above, mix cubes thoroughly, and remove subsamples to waste, mixing after each removal, until only a few grams remain. Remove 1 g sample, or preferably two or three 1 g samples for replicate analyses. Measure sample(s) in pre-calibrated volumetric containers, e.g., small plastic beakers. Alternatively, for either type sample, use portable balance intended for field use, e.g., Ohaus® Model 10-10.

Determination

Add 5 mL acetone to sample in hand-operated homogenizer. Mix at least 20 min to obtain good homogenate. If electricity is available, small stirring motor or electric drill motor (some have cordless power packs) mounted on ring stand can be used to spin homogenizer pestle (active part) while tube is moved by hand into strategic positions, thus shortening time for homogenization to less than 10 min.

Decant all acetone extract into screw-cap test tube. Add 2 mL each of hexane and dichloromethane, cap tube, and mix contents thoroughly for 1 min. Let settle, then use dropping pipet to transfer to clean 100 mL beaker as much as possible of upper layer, without disturbing small lower layer. Add sodium sulfate to cover bottom of beaker and swirl beaker so that all liquid contacts sodium sulfate. Place beaker on outer edge of warm, unplugged hot plate or, similarly, on warm aluminum cover plate of campstove, with all burners turned off. If boiling is too vigorous, move beaker nearer edge or remove it until hot plate or cover plate has cooled. When solvent has just dissipated from any beaker, add 5 mL hexane from 10 mL syringe with needle. Let solvent evaporate again and this time remove beaker from heat. Add an additional 3 mL hexane, and swirl to dissolve residues. Take up hexane solution in clean syringe with needle. Exchange needle for Sep-Pak Florisil cartridge that has been prerinsed with 5 mL hexane, and expel syringe contents slowly through cartridge into waste jug. Add another 2 mL hexane to beaker, swirl, and similarly take up in same syringe and expel this through cartridge. Follow similarly with 5 mL 6% ether in hexane. Then add 5 mL 15% ether in hexane, and collect all cartridge effluent in flat-bottom screw-cap vial. (If desired, remove 1 mL aliquot and save in labeled screw-cap vial for possible later analysis.) Add several crystals of sodium chloride, then evaporate solvent as described above. Add 0.5 mL 2% 4-(*p*-nitrobenzyl)pyridine with 0.08% oxalic acid in acetone. Evaporate acetone, with hot plate unplugged or stove burners off during evaporation as mentioned above. Caution: These solvents are very flammable. Now remove samples and start heat source so that salt bath can be regulated to 150°C. Place samples in bath and heat 3 min at 150°C, then remove samples and let cool. In orderly, timed sequences, add 1.0 mL TEA reagent (20% triethylamine in acetone stored in brown bottle), and then 0.4 mL Na_2CO_3-NaCl solution (12% Na_2CO_3 and 15% NaCl in water). Swirl gently to mix, let settle, and transfer by dropping pipet sufficient clear upper phase to a microcell, and read, within 3 min after mixing, at 560 nm vs instrument set at 100% *T* with acetone. Dispose of test solution in waste jug and rinse cell with acetone from polyethylene wash bottle, letting rinsings collect through funnel into waste jug.

For best quantitation, compare unknown samples with fortified control standards similarly carried through method. This approach simultaneously corrects for background response in samples while providing a sound quantitative basis. Once an analyst is able to perform tests with satisfactory accuracy and reproducibility from day to day, it is not necessary to prepare standard graph each day. Instead, a replicate fortified level should be run each day to confirm that method is still giving satisfactory results.

Results and Discussion

Citrus rind samples (1 g) from control fruit consistently gave no positive color with the method, and absorbance readings were always within 0.18–0.26 absorbance unit vs acetone. These were not much different from reagent blank runs of 0.16–0.17 absorbance unit.

Citrus rind fortified with parathion in the laboratory at the time of extraction at levels corresponding to 1 ppm (whole fruit basis) or greater gave positive color reactions with absorbance readings of greater than 0.4 unit.

Replicate samples from lemons, from which residues of previously sprayed parathion had largely dissipated, were carried through this method and aliquots from the cleaned extracts were taken for gas chromatography (GC) and liquid chromatography (LC) (UV 265 nm). (Controls gave no interferences in the retention time region for parathion for GC with either

electron capture or alkali flame ionization detection (AFID); the latter mode gave the cleanest chromatograms.) Color reactions carried out on the bulk of the cleaned extracts were negative, with absorbance readings of 0.21 and 0.22 unit. Both LC and GC analyses produced readily measured peaks corresponding to parathion retention times and provided calculated levels of 0.7 and 1 ppm, respectively, on or in the rind. These results correspond to between 0.2 and 0.3 ppm on or in the whole fruit, well below the U.S. tolerance level of 1 ppm (whole fruit basis). The color test method would have correctly approved these lemons for harvesting if the testing had been conducted for that purpose. Previous research here has established that essentially no parathion penetrates into the pulp of citrus (3).

To simulate field-sprayed conditions, a section of rind from a control navel orange was soaked 8 h in a parathion wettable powder solution (0.72 g/400 mL water) and then air-dried several days in a fume hood. From similarly air-dried control rind sections, we determined that air-drying caused a loss of 70% of the rind weight. Therefore, 0.3 g samples of control and test rind samples, equivalent to 1 g each of freshly peeled rind, were individually carried through the entire method. The control produced a reading of 0.20 absorbance unit; the reading from the test rind sample was 0.58 unit.

The method described is more specific for parathion (ethyl or methyl) compared with its specificity for other potential organophosphorus pesticide interferants. To interfere, a compound must have adsorptivity on Florisil similar to that of parathion so that it will elute from a Sep-Pak Florisil cartridge under the same conditions. This excludes most other organophosphorus pesticides with the known exceptions of diazinon, dioxathion, and ethion. None of these is presently in use in California citriculture.

We believe that the method can be extended to apples. A 1 g sample of pulp and peel from a commercial Golden Delicious apple gave an absorbance reading of 0.18. GC analysis with AFID gave no background response on an aliquot from the same sample.

A field test kit to carry out this method can be readily assembled; Sep-Pak cartridges lend themselves especially well to this purpose. The Bausch and Lomb mini Spectronic 20 spectrophotometer was specified earlier for use in a field method for determining anti-cholinesterase insecticide spray deposits on plant surfaces (4).

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Automated Extraction Technique for Determination of Experimental Insecticide Nifluridide and Its Cyclized Product in Water by High Pressure Liquid Chromatography

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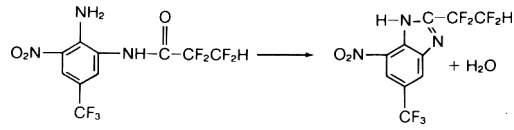
A method is described for the simultaneous determination of the experimental insecticide nifluridide (*N*-(2-amino-3-nitro-5-(trifluoromethyl)phenyl)-2,2,3,3-tetrafluoropropanamide) and its cyclized product, EL-919 (7-nitro-2-(1,1,2,2-tetrafluoroethyl)-5-(trifluoromethyl)benzimidazole), in water. Both compounds are extracted by passage of up to 500 mL water through two Sep-Pak C_{18} cartridges joined in series. The extraction is automated by using vacuum. The compounds are eluted from the cartridges with acetonitrile. Both compounds are then separated and measured by high pressure liquid chromatography with UV detection at 235 nm. Recoveries were 91.2-97.1% nifluridide and 82.7-98.0% EL-919 for deionized water samples fortified with 0.001-0.1 ppm nifluridide and 0.005-0.1 ppm EL-919. Analysis of water samples (pH 8) from bluegill and rainbow trout toxicity studies with nifluridide resulted in a half-life determination of 10 h at 13°C and 2.5 h at 20°C with nearly quantitative conversion to EL-919 within 96 h.

Nifluridide (*N*-(2-amino-3-nitro-5-(trifluoromethyl)phenyl)-2,2,3,3-tetrafluoropropanamide) is an experimental insecticide that is being evaluated for the control of the imported fire ant (*Solenopsis germinata*) and the red imported fire ant (*S. invicta*) in the southern United States. Broadcast applications of formulated ant baits containing 0.75% nifluridide at rates of 10–20 g active ingredient/ha have resulted in delayed toxicity to ants, which permits distribution of the material throughout the colonies (1, 2).

A previous study (3) has shown nifluridide to be unstable in aqueous solutions, with rapid conversion to the cyclized product EL-919 (7-nitro-2-(1,1,2,2-tetrafluoroethyl)-5-(trifluoromethyl)benzimidazole). The conversion proceeded as indicated in Figure 1 via a first-order reaction at 25°C with half-lives of 15.5, 3.5, and 2.0 h at pH 5.0, 7.0, and 9.0, respectively (3). The instability of nifluridide has presented problems for its determination in water from studies conducted to determine its toxicity to fish and aquatic organisms. Because rapid cyclization of nifluridide would be expected to occur before the water samples could be sent to the laboratory and analyzed, it would not be possible to accurately determine the initial exposure concentrations in the water. Consequently, it was necessary to develop a method that would stabilize nifluridide as the parent compound until its concentration could be determined. In this paper, a method is described for stabilizing nifluridide for the simultaneous determination of both the parent compound and its cyclized product in water from environmental toxicity studies. The method employs a vacuum apparatus to automate the extraction of the chemicals in water samples onto Sep-Pak C_{18} cartridges. Because of the hydrophobic nature of nifluridide and EL-919, the compounds are adsorbed onto the octadecyl silane bonded phase of the cartridges and then eluted with a small volume of acetonitrile.



NIFLURIDIDE EL–919

Figure 1. Conversion of nifluridide to EL-919.

METHOD

Apparatus

(a) *Sep-Pak C_{18} cartridges.*—Waters Associates.

(b) *Sep-Pak extraction apparatus.*—Figure 2.

(c) *Glass syringe.*—20–50 mL with Luer tip.

(d) *High pressure liquid chromatograph.*—Varian Model 5060 pump, Varian Model 8055 autosampler, Dupont variable wavelength UV spectrophotometer, and strip chart recorder. Pump methanol–water–acetic acid (65 + 35 + 0.1) mobile phase through CO:PELL ODS guard column (Whatman, Inc.) and Lichrosorb RP18 column at 1.5 mL/min. Injection volume 25 μL; operate detector at 235 nm and 0.02 AUFS.

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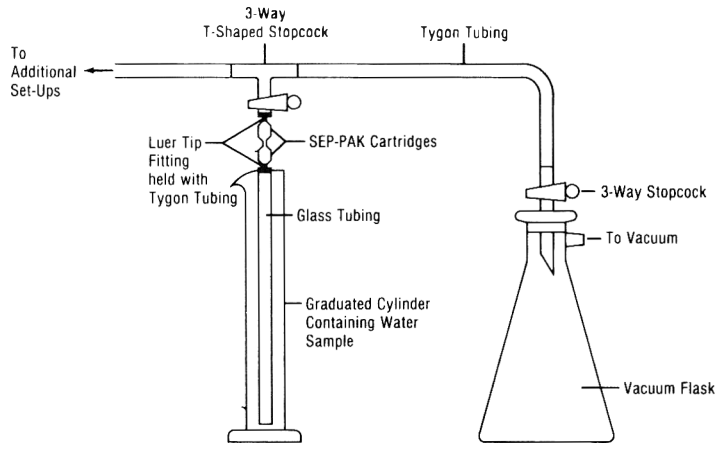

Figure 2. Vacuum extraction apparatus for automatically extracting several large-volume water samples with cartridges.

Reagents

(a) *Solvents.*—Acetonitrile, methanol, and water (all HPLC grade); glacial acetic acid (reagent grade).

(b) *Standard solutions.*—(1) *Stock solution.*—1.0 mg nifluridide plus 1.0 mg EL-919/mL acetonitrile. (2) *Intermediate solution.*—100 μg nifluridide plus 100 μg EL-919/mL acetonitrile. Transfer 5.0 mL stock solution to 50 mL volumetric flask and dilute to volume with acetonitrile. (3) *Direct standard.*—2.0 μg nifluridide plus 2.0 μg EL-919/mL acetonitrile. Transfer 1.0 mL intermediate solution to 50 mL volumetric flask and dilute to volume with acetonitrile. Prepare direct standards at other concentrations, if appropriate. Both compounds are stable in acetonitrile under refrigeration for at least one month.

Automated Sample Extraction

Using short piece of Tygon tubing, join short end of one Sep-Pak C_{18} cartridge to long end of second cartridge. Place ends of cartridges in close contact with one another to prevent contact of water sample with Tygon tubing. Use metal Luer tips to attach long end of resulting dual cartridges to glass tubing leading to graduated cylinder, and attach short end to 3-way T-shaped stopcock (Figure 2). Rinse cartridges by pulling 20 mL acetonitrile through cartridges under vacuum, followed by 20 mL rinse with deionized water. Discard rinses. Measure required volume (up to 500 mL) of water sample in graduated cylinder. Using vacuum, pull water sample through dual cartridges at ca 15–20 mL/min until both cartridges are essentially free of residual water. Discard water. Detach cartridges from extraction apparatus, and attach long end of joined cartridges to Luer tip of glass syringe. Elute nifluridide and EL-919 by pumping 20 mL acetonitrile through cartridges into 125 mL evaporating flask. Evaporate acetonitrile eluate to dryness on rotary vacuum evaporator with

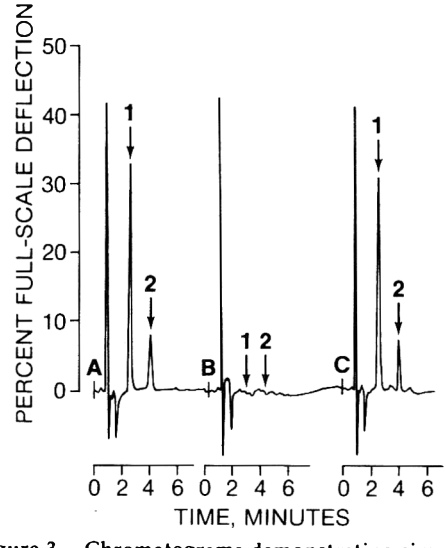

Figure 3. Chromatograms demonstrating simultaneous determination of nifluridide and EL-919 in well water from fish tanks: A, standard nifluridide plus standard EL-919, 62.5 ng each; B, control well water extract; C, control well water fortified with 25 ppb nifluridide (93.4% recovery) and 25 ppb EL-919 (92.7% recovery).

Table 1. Recovery of nifluridide and EL-919 from water

Fortified, ppm	Sample vol., mL	Nifluridide		EL-919	
		N	% Rec.	*N*	% Rec.
0.100	100	6	95.8 ± 2.0	5	90.1 ± 2.8
0.025	200	6	91.2 ± 1.6	6	88.8 ± 3.7
0.001	500	6	97.1 ± 5.1	6	ND[a]
0.100	100	0		6	82.7 ± 2.9
0.025	200	0		5	97.6 ± 4.0
0.005	500	0		5	98.0 ± 4.1

[a] None detected.

water bath heated to 35–45°C. Dissolve residue in measured volume of acetonitrile (use 1.0 mL for maximum assay sensitivity). Quantitate both compounds simultaneously by HPLC.

Results and Discussion

For the determination of nifluridide concentrations in water from environmental toxicity studies with the insecticide, a method that stabilized nifluridide for analysis was desirable. Nifluridide does not cyclize to form EL-919 in acetonitrile; therefore elution of the compound from Sep-Pak cartridges with acetonitrile stabilized the insecticide as the parent compound. Thus, both compounds could be extracted from water onto Sep-Pak C_{18} cartridges, eluted with acetonitrile, and sent to the analytical laboratory as the acetonitrile extract. At the laboratory, the extract was stored under refrigeration until analyzed. Nifluridide and EL-919 are stable in acetonitrile under refrigeration for at least one month.

Both compounds were separated and quantitated on the same chromatogram, as shown in Figure 3. The absence of interfering peaks in the chromatogram of the control water extract demonstrates that the method possesses adequate selectivity for determining the insecticide and its cyclized product in water from fish tanks.

The automated extraction technique was used to facilitate the extraction of large volumes of water through the cartridges, as opposed to manually pumping water through cartridges with a glass syringe (4–7). With the apparatus shown in Figure 2, several large-volume water samples may be processed automatically. We have found that this technique may also be used for laboratory extraction of many other compounds from aqueous solution. With some compounds (including EL-919), it is necessary to join 2 cartridges to increase recoveries.

The apparatus was designed to prevent extraction of interfering substances such as phthalates from the Tygon tubing. As shown in Figure 2, the compounds of interest are extracted from the water onto the cartridges before the water passes through the long section of tubing leading to the vacuum flask, which also serves as a wastewater collection container. The water entering the cartridges from the graduated cyl-

Table 2. Conversion of nifluridide to EL-919 in water at 13°C and 20°C

Water temp., °C	Concn range, ppm[a]	Time, h	% of Initial (av. ± SD)	
			Nifluridide	EL-919
13	0.3–1.6	0	91.2 ± 13.8	8.8 ± 13.8
		6	57.5 ± 21.8	24.2 ± 12.7
		24	34.0 ± 9.9	77.8 ± 10.1
		48	10.1 ± 2.8	93.0 ± 15.7
		96	0.8 ± 1.3	86.2 ± 30.9
20	0.16–0.45	0	90.8 ± 4.9	9.2 ± 4.9
		6	24.3 ± 5.2	55.5 ± 11.1
		24	ND[b]	83.5 ± 5.0
		48	ND	78.2 ± 5.4
		96	ND	81.3 ± 6.0

[a] At 13°C, nominal concentrations were 0.3, 0.4, 0.56, 0.8, 1.1, and 1.6 ppm. At 20°C, nominal concentrations were 0.160, 0.200, 0.250, 0.300, 0.365, and 0.450 ppm.

[b] None detected.

inders contacts the glass tubing and metal Luer tips but does not contact the Tygon tubing that stabilizes the connections.

The method described in this paper is capable of determining concentrations as low as 0.001 ppm nifluridide and 0.005 ppm EL-919. The recovery levels and precisions obtained by analyzing replicated samples of deionized water fortified with both compounds or with EL-919 alone are summarized in Table 1. These recoveries demonstrate the stability of nifluridide during the extraction process.

This method was used for analyzing water samples (pH 8) from environmental toxicity studies with rainbow trout and bluegill, and assay results are summarized in Table 2. The half-life for nifluridide was determined to be approximately 10 h at 13°C and 2.5 h at 20°C, with nearly quantitative conversion to EL-919 within 96 h. The half-life of 2.5 h at pH 8 and 20°C correlated well with the previously determined half-life of 2.0 h at pH 9 and 25°C obtained by direct injection HPLC (3).

During the course of analysis of water samples from fish toxicity studies, recovery data were also obtained by fortifying control well water with both compounds. No significant differences in recoveries were observed with the well water samples compared with those with deionized water.

Acknowledgments

The authors thank R. L. Cain and R. O. Burger for technical assistance.

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FOODS

Improved Method for Determination and Identification of Serotonin in Foods

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A previously described method to identify and quantitate serotonin in foods has been improved. The extraction and separation of serotonin from interfering substances has been improved, and the scope of material to which the method may be applied has been widened. The relative standard deviation (RSD) for repeated determinations of serotonin in canned fried tomato purée and the average recovery of serotonin added to the same sample was 6.25 and 89.9%, respectively. The method showed the presence of serotonin in apricots, cherries, and peaches.

In a previous paper a method was described for the determination of serotonin in foods (1). The method included alkaline butanol elution from a sand column, extraction of serotonin from the eluate with 0.1N HCl, quantitative determination by spectrofluorometry, and verification by spectrofluorometry and thin layer chromatography (TLC). This method was satisfactorily applied to fresh bananas, banana-based baby foods, and fresh and canned tomatoes.

There is great interest in the possible presence of serotonin in other foods because of its biological activity, direct toxicity, interaction with certain drugs, and even possible beneficial effects (2–7). Therefore, we attempted to extend this method to more complex derivatives of the foods studied (canned fried tomato purée, for example) and other foods: apricots, cherries, plums, and peaches. However, when the procedure was applied to samples of such foods, the fluorescence spectra of food extracts were not the same as those of standard serotonin. In addition, TLC results were not definitive in spite of using lead acetate as a clarifier, although serotonin apparently was detected.

It may be deduced that different compositions of food give rise to the presence in the final extracts of interferences which were not present in the samples initially studied. Therefore, we studied the effects of some procedural modifications to improve the original serotonin assay: (a) mixing the sample with anhydrous sodium sulfate to obtain a more selective extraction and avoid the interference of water; (b) treating the sample before extraction with serotonin-insoluble organic solvents; (c) treating the butanol eluate with other solvents after extraction and before carrying out spectrofluorometry; and (d) treating the final acid solution to remove interfering substances before qualitative verification by TLC.

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METHOD

Apparatus

(a) *Spectrofluorometer.*—Aminco SPF 125.

(b) *Rotovapor.*—Buchi R.A. with W-240K bath.

(c) *TLC plates.*—For 5 plates, 20 × 20 cm, homogenize 35 g silica gel G (type 60; Merck) and 70 mL water. Activate plates 30 min at 110°C before use.

(d) *Glass columns.*—500 × 300 mm, with No. 0 filter in bottom, and stopcock (Afora).

Reagents

(a) *Serotonin-creatinine sulfate monohydrate.*—(Merck). Used throughout study, with concentrations expressed as function of pure serotonin.

(b) *Alkaline butanol.*—Mix a few drops (ca 1 mL) of saturated aqueous solution of NaOH with 200 mL analytical reagent grade *n*-butanol, to pH 9–10.

(c) *o-Phthalaldehyde (OPT).*—Dissolve 0.5 g OPT (Fluka) in 100 mL ethanol.

Calibration Curve

Prepare solution of 1 mg serotonin/mL 0.1N HCl. Prepare fresh every day. Pipet 1 mL this solution into 100 mL flask and dilute to volume with 0.1N HCl (10 μg/mL), and from this, prepare solutions containing 0.01, 0.02, 0.06, 0.10, 0.40, 0.60, 1.00, 1.50, and 2.00 μg/mL 0.1N HCl.

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Read excitation wavelength at 295 nm and emission wavelength at 340 nm against 0.1N HCl blank. Obtain new calibration curve for each series of determinations.

Extraction and Separation

Homogenize samples in mechanical blender. Weigh 15 g sample and place in 200 mL beaker, and then add fine sand and anhydrous Na_2SO_4 (2 + 1) until resulting mixture is practically dry, that is, consistency should be dry and should not form lumps (ca 90 g sand and 45 g anhydrous Na_2SO_4). Add 50 mL alkaline butanol and let stand 0.5 h, shaking occasionally. Pack this mixture into glass column which is shielded with black paper (serotonin is sensitive to light). Wash beaker with 150 mL alkaline butanol and add wash to column.

Extraction proceeds as drop-by-drop elution of butanol from column. Adjust elution to last ca 1 h. Extract alkaline butanol eluate in separatory funnel 6 times with 20 mL portions of deionized water. Add 10 mL petroleum ether to water-extracted butanol eluate. Extract washed butanol eluate with five 20 mL portions of 0.1N HCl, combine 0.1N HCl extracts, and adjust with 0.1N HCl to a final volume of precisely 100 mL.

Spectrofluorometry

Quantitative analysis.—Read fluorescence values of HCl solution at maximum excitation wavelength 295 nm and maximum emission wavelength 340 nm.

Qualitative analysis.—Compare excitation and emission spectra of HCl solution to ensure that they coincide with those of pure serotonin.

Thin Layer Chromatography

Wash HCl solution (obtained by extraction and separation procedure) 3 times with 20 mL ethyl ether each time. Evaporate ether-extracted 0.1N HCl solution to dryness in rotovapor at 40°C and redissolve in 1 mL 0.1N HCl. On same TLC plate, spot standard serotonin, sample extract, and serotonin standard and sample superimposed. Develop plate with chloroform–methanol–acetic acid (70 + 20 + 40).

Spray with OPT; after spraying, subject to heat (110°C) for 20 min. Serotonin gives characteristic brown-yellow fluorescence at 360 nm.

Results and Discussion

To verify the reproducibility of the improved method, serotonin was determined 10 times on different days on the same batch (canned fried tomato purée). Results were 1.6, 1.7, 1.5, 1.6, 1.6, 1.4, 1.5, 1.7, 1.6, and 1.7 ppm. The relative standard deviation (RSD) was 6.25% (8).

Canned fried tomato purée was chosen because of the complexity of its ingredients and the technological processes it undergoes. Salt is added to tomato pulp and the mixture is fried in vegetable oil before sterilization and canning. All other samples tested lacked interferences.

To determine percent recovery, different amounts of serotonin, 0.5, 1.0, 2.5, 5.0, and 10.0 ppm, were added to a single sample and assayed 5 times at each level (Table 1). Average recovery of the 25 assays was 89.9%.

When the original and improved methods were applied to a single sample, only the fluorescence spectrum of the latter corresponded to serotonin (Figure 1). When the aqueous phase (obtained by washing the butanol phase) was dried and redissolved in 0.1N HCl, the fluorescence spectrum of this solution corresponded to the spectrum for possible interfering substances, as may be deduced from a comparison with the spectrum obtained with the original method.

The method was satisfactorily applied to apricots, cherries, plums, peaches, and canned fried tomato purée, regarding the absence of interferences and recoveries. Recoveries were in the same range as those recorded for tomato purée. Recoveries were verified by addition of serotonin standard, and the presence of seroto-

Table 1. Recovery (%) of serotonin added to different samples of the same canned fried tomato purée

Added, ppm	Rec., %[a]					Av. rec., %
0.5	80.0	100.0	100.0	80.0	80.0	88.0
1.0	90.0	100.0	90.0	90.0	80.0	90.0
2.5	80.0	88.0	92.0	84.0	100.0	88.0
5.0	90.0	94.0	82.0	96.0	98.0	92.0
10.0	93.0	94.0	92.0	95.0	80.0	90.8
Overall av. rec.						89.9

[a] In all cases, the amount of serotonin originally contained in the sample was taken into account.

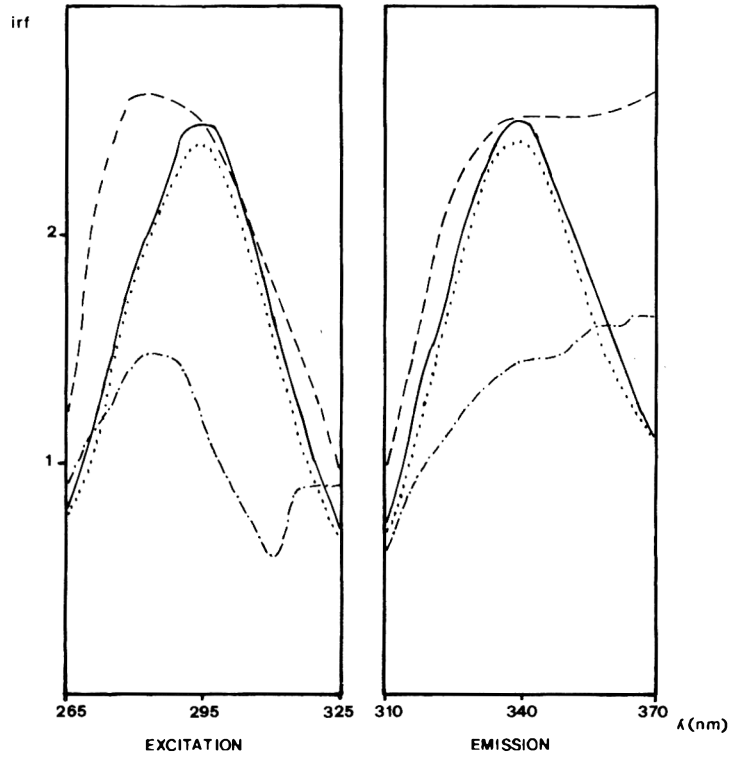

Figure 1. Fluorescence spectra of serotonin standard solution and final extracts obtained by applying original and improved methods to a single sample (canned fried tomato purée). _____ Serotinin; - - - - - - without washing alkaline butanol phase (original method); ····· after washing alkaline butanol phase (improved method); -·-·-·-·- liquid used for washing.

nin was confirmed by TLC. As far as we know, the presence of serotonin has not been described previously in any of the materials in which we have found it: apricots, cherries, and peaches (9).

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FISH AND OTHER MARINE PRODUCTS

Identification of Frozen, Cooked Shellfish Species by Agarose Isoelectric Focusing

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A modification of the AOAC official method for generic identification of cooked and frozen crabmeat was investigated in an experiment in which the cooked meats of a variety of shellfish were identified. The modification, substituting agarose for polyacrylamide as the gel medium, has many advantages over the official method, including ease of gel preparation, nontoxic reagents, and rapid focusing. Results indicate that the modified method is easier to use and that identifications of cooked shellfish species can be made as readily as with the current AOAC method.

Species of crabs in cooked, frozen crabmeat have been successfully identified using urea-based polyacrylamide gels for isoelectric focusing (PGIEF) as described by Krzynowek and Wiggin (1, 2). Focusing times of 18–24 h with the previous method were relatively lengthy, and polyacrylamide gels contain the toxic acrylamide monomer (3). The introduction of a purified agarose gel enables researchers to use a stable, nontoxic medium for isoelectric focusing. It has essentially no reactive groups and gels quickly without a catalyst. It has been shown that agarose gel isoelectric focusing (AGIEF) gives reproducible species-specific sarcoplasmic protein patterns in species of raw fish (3). This method was modified to focus the cooked proteins that are encountered in our species identification studies of cooked products. This paper reports on those changes and the use of AGIEF as a substitute for the previous PGIEF method of species identification. Species investigated include 6 genera of crab: red (*Geryon quinquedens*), Jonah (*Cancer borealis*), blue (*Callinectes sapidus*), king (*Paralithodes camtschatica*), snow (*Chionoectes spp.*), and stone (*Menippe spp.*). Blue mussels (*Mytilus edulis*), surf clams (*Spisula solidissima*), spiny lobster (*Panulirus argus*), and rock shrimp (*Sicyonia spp.*) were included to show that they also focus into unique, species-specific patterns.

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Experimental

Apparatus

(a) *Electrofocusing apparatus.*—Pharmacia flat bed apparatus FBE 3000 (Pharmacia Fine Chemicals, Piscataway, NJ 08854).

(b) *Power supply.*—Pharmacia electrophoresis constant power supply ECPS 3000/150.

(c) *Slab cooling system.*—Neslab bath cooler PBC-4 and Neslab Tamson circulator (Neslab Instruments, 871 Islington St, Portsmouth, NH 03801).

(d) *Sample application foil.*—Pharmacia Cat. No. 19-2942-01 or FMC Corp. Lot No. 99289.

(e) *Sample applicator papers.*—3MM Whatman paper cut to ca 5 × 10 mm.

(f) *Gel backing.*—"GelBond," 7 mil: polyester support treated on one side for adherence of gel (FMC Corp., Marine Colloids Div., Rockland, ME 04841).

(g) *Filter paper.*—S&S No. 577 (Schleicher & Schuell, 543 Washington St, Keene, NH 03431).

Reagents

(a) *Electrolytes.*—0.1M NaOH (catholyte); 0.1M H_3PO_4 (anolyte).

(b) *10M urea.*—Schwarz/Mann (Mountain View Ave, Orangeburg, NY 10962) ultra pure urea. Prepare new solution each day.

(c) *Ampholytes.*—LKB Ampholine, pH 4–6, pH 3.5–5, pH 3.5–10 (LKB Instruments Inc., Rockville, MD 20852).

(d) *Agarose.*—Marine Colloids IsoGel.

(e) *Sorbitol.*—20% (w/v).

(f) *Fixative.*—4% trichloroacetic acid and 3% sulfosalicylic acid in methanol–water (30 + 70).

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(g) *Clearing solution.*—Water–ethanol–acetic acid (67 + 25 + 8).

(h) *Stain.*—0.2% Coomassie Brilliant Blue R-250 or Serva Violett 49 in clearing solution.

Preparation of Samples

All samples were composites of at least 10 shellfish of any one species. The shrimp sample was a composite of 40 shrimp. Previous studies show no pattern differences among individuals of any one species.

All samples were cooked in boiling water to extract the meat, and subsequently frozen to −20°F. Jonah crabs were boiled in 3% NaCl for 15 min and the meats were handpicked and then frozen. The remaining crabmeat samples were purchased from commercial sources already processed and frozen. Mussels were harvested, cooked until gaped, shucked, and stored frozen in plastic tubs. The rock shrimp and spiny lobster were sent frozen raw from Florida. The rock shrimp were simmered 1 min, and the lobster, 20 min. They were then handpicked and frozen. Surf clams were aquaculturally grown in Milford, CT. They were steamed over boiling water 8 min, shucked, and stored frozen in plastic tubs. In all cases, cooking methods were "cookbook" recommendations. No pattern differences were observed among different batches of the same species if the same cooking times were adhered to. Longer cooking times can result in fainter protein banding patterns after staining, but the pattern itself remains unchanged. Before sampling, both the mussel samples and clam samples were each thawed and blended until homogeneous. An aliquot was used for protein extraction. All samples were blended with 10M urea in a ratio of 2:1 (wt:vol.) meat:urea for approximately 2 min. The resulting mixture was centrifuged at 12 800 g, and the supernate was used for spotting. Amounts as small as 0.8 g meat to 0.4 mL urea may be used.

Preparation of Gel

The gel was made according to Lundstrom (3). A 30 mL syringe holds enough gel solution for 2 gels, 105 × 200 × 0.75 mm thick. The gel mold is made by rolling a piece of GelBond (hydrophilic side towards gel), cut to the same dimensions as the glass plates (105 × 200 mm), over a small amount of water on one glass plate. A 0.75 mm thick spacer bar is placed on the GelBond, followed by a glass plate. The entire assembly is clamped together on 3 sides. The mold and the syringe must be warm (under an infrared lamp) while pouring the gel. Then 0.24 g IsoGel is mixed in 15 mL water and heated to 90°C to dissolve; 13.5 mL 20% (w/v) sorbitol is added, mixed, and cooled to about 70°C before adding 1.0 mL Ampholine, 40% (w/v), pH 4–6; and 0.5 mL Ampholine, 40% (w/v), pH 3.5–10. Mixed solution is poured into 2 molds, using syringe. Gel blanks (IsoGel in water and heated to 90°C) may be made ahead of time and stored at 4°C. Gels have been stored in their molds at 4°C for 2 days with no apparent ill effects. The open end must be sealed with tape to prevent dehydration. The agarose gels in ca 20 min and is then refrigerated ≥30 min.

Procedure

When ready to use gel, carefully separate glass plates and remove spacer bar, leaving gel adhered to GelBond. Place GelBond over a small amount of water on cooling slab, working out air bubbles to ensure good contact with cooling platform. Maintain coolant at 0–10°C. Blot gel with S&S No. 577 filter paper, cut to size of gel, to remove excess water. Place sample application mask or 3MM Whatman paper (sample applicator papers) at cathode end. Pipet either 5 or 10 μL sample supernate into mask, depending on size of loading slit. Allow about 5 min for gel to absorb sample, and then remove mask. If using sample applicator papers, pipet 10–20 μL sample supernate onto papers, where it is absorbed immediately. Place electrolyte wicks soaked with anolyte and catholyte solutions at ends of gel; place platinum electrode wire on top of wicks. Focus proteins 30 min by applying 30 watts of constant power with a limiting voltage of 1500 V and a limiting current of 50 mA. When using sample applicator papers, remove these after 15 min and continue separation for another 15 min.

Fixing, Staining, and Drying

After focusing time is complete, remove electrolyte wicks from gel and place gel in fixative 15 min. Remove gel from fixative and place wet piece of S&S No. 577 filter paper over entire gel. Lay gel, filter paper side down, over stack of paper towels. Place glass plate and 500–1000 g weight on top. Remove saturated paper towels to speed drying process. After 15 min, carefully peel off filter paper and dry gel under infrared lamp until white protein precipitates are no longer visible and gel is dry to touch. Place gel in clearing solution 1 min, and then in stain heated to 60°C for ca 1 h. Destain briefly in 2–3 changes of clearing solution. Again, dry gel under infrared lamp.

Results and Discussion

We have found several procedural techniques with this method that yield protein band patterns that stain darker and are more unique to the species. One such procedural technique would be the choice of matrices for the gel medium. Although the incorporation of urea into polyacrylamide gels greatly enhanced the protein bands (Figure 1) for that medium, agarose gels gave better results if urea was omitted and gels were made with water. Urea appeared to weaken the strength of the agarose gel, making it watery. Urea-based gel did not adhere well to the GelBond, and dense background staining made the protein bands hard to distinguish. Exclusion of urea from the gel allowed raising the power from 1 watt constant power in overnight focusing as in the PGIEF method to 30 watts for 30 min in this method.

Another deviation from the PGIEF method (Figure 2) was the placement of extracted protein at the cathode rather than the anode and the use of acidic pH ranges for the focused gradient. Comparison of the 2 plates done by the 2 methods shows an area of precipitated protein in the AGIEF method which is prevented in the PGIEF method by the inclusion of urea in the polyacrylamide gel.

Samples extracted with 10M urea gave the darkest banding patterns after staining. Urea (10M) was more successful as an extracting solution (Figure 3) than 2% Triton X-100 (non-ionic detergent), 1% and 10% sodium dodecyl sulfate (SDS) (anionic detergent), 10% glycine, or 20% diethylene glycol. These were blended with the sample meat in a ratio of 1:1 (wt:vol.) or 2:1 as with the urea. Sample meat left in contact with 10M urea for more than 1 day and centrifuged before spotting produced darker stained patterns than freshly extracted and centrifuged supernate (Figure 4). Extracted samples have been frozen for 2 weeks with no change in their banding patterns. The extraction step greatly affects the amount of stainable material after focusing. While close inspection of the focused and stained plates reveals that all bands are present and reproducible from plate to plate for any one species, the variation in staining intensities can

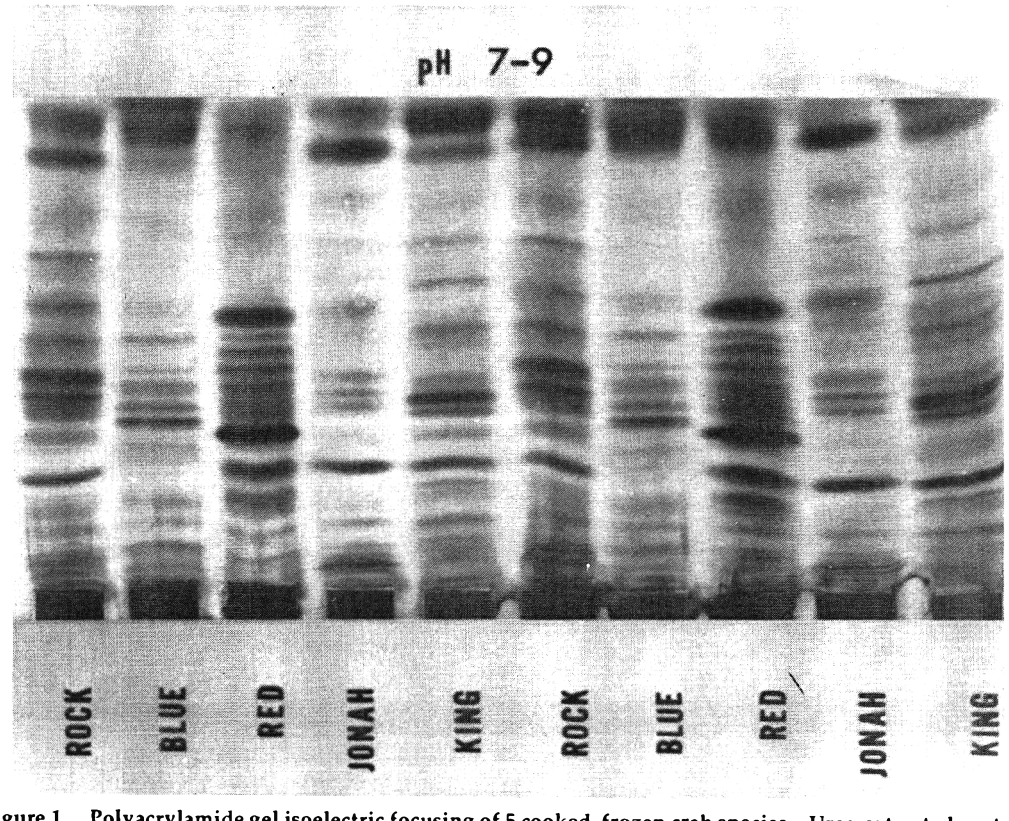

Figure 1. Polyacrylamide gel isoelectric focusing of 5 cooked, frozen crab species. Urea-extracted protein (20 μL) was spotted at anode on 3MM Whatman paper.

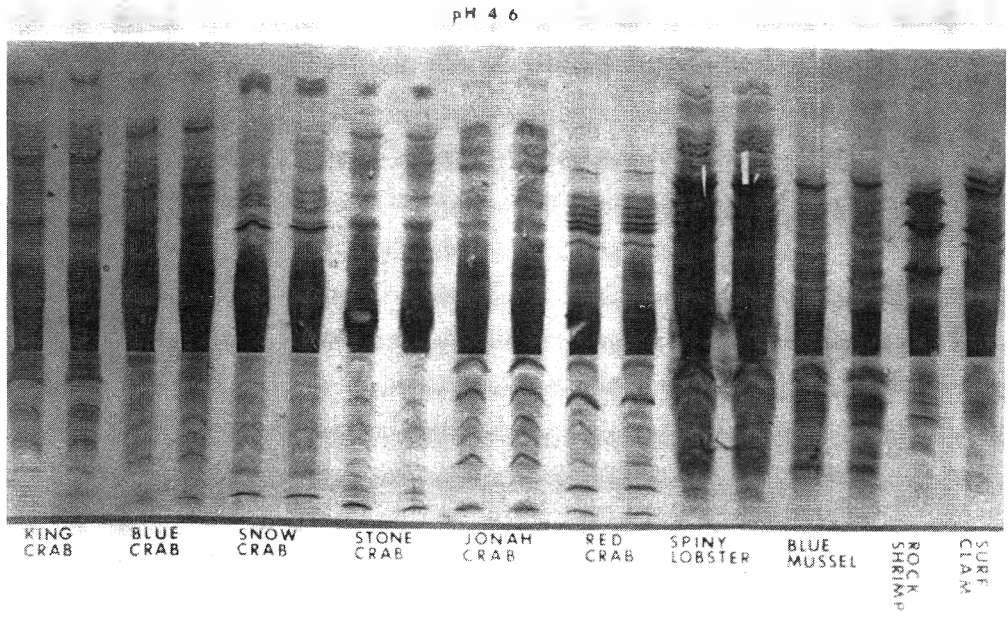

Figure 2. Agarose gel isoelectric focusing of 10 cooked, frozen species. Urea-extracted protein (10 μL) was spotted at cathode. Dark areas are precipitated proteins.

lead to erroneous sample identification, as might occur in Figure 4 between 1-day-old and 5-day-old red crab. For this reason, it is suggested that authenticated samples be run simultaneously and identically with unknown samples.

This method offers an excellent alternative to PGIEF. The main advantage of the PGIEF method is the incorporation of urea into the gel,

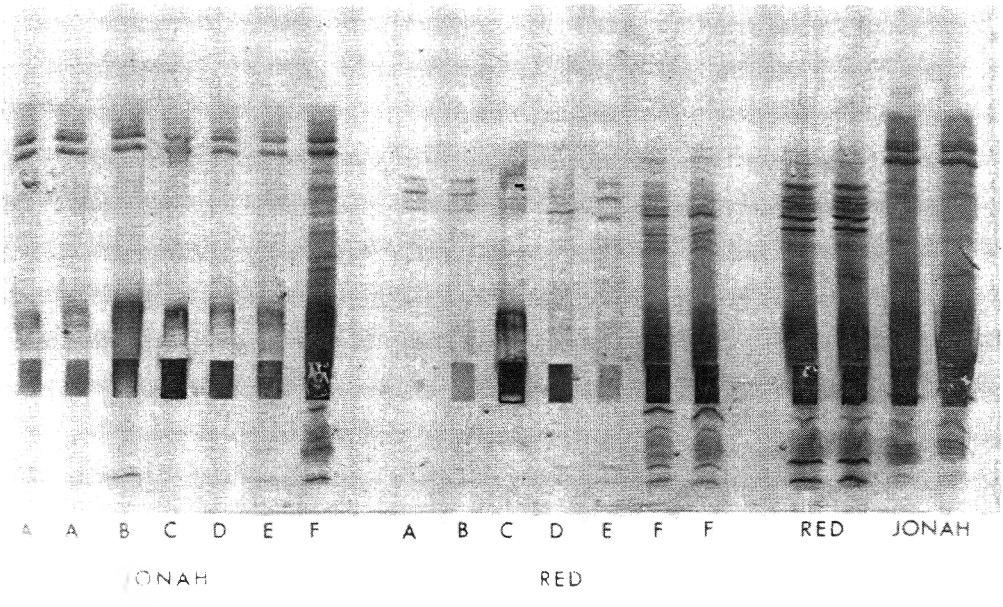

Figure 3. AGIEF showing different extraction procedures. Samples supernate (10 μL) was spotted at cathode in loading slits of plastic mask. A, 20% diethylene glycol; B, 10% glycine; C, 10% SDS; D, 1% SDS; E, 2% Triton X-100, and F, 10*M* urea. The latter 4, red and Jonah crabs, were urea-extracted 30 days before spotting on this plate (pH 4-6).

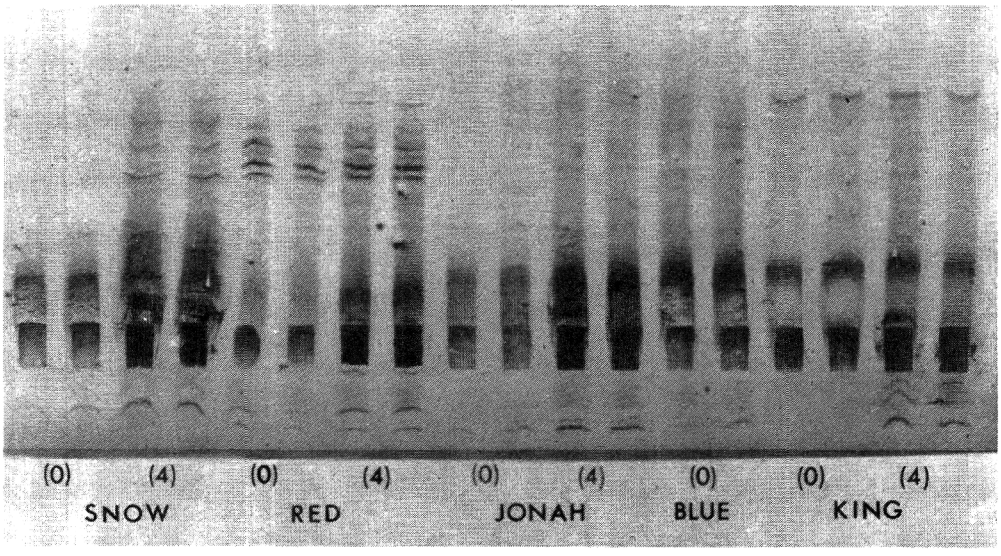

Figure 4. AGIEF showing 5 crab species in pH 4-6 range and spotted at cathode. The first 2 in each set of 4 were freshly extracted supernate, and the second 2 were extracted 4 days before spotting on this plate.

which keeps the denaturated proteins in solution in the gel. Although proteins precipitate near the cathode in the AGIEF method, this method is certainly more rapid and safer than PGIEF, and species of cooked products can be identified.

Acknowledgments

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Fish Species Identification by Agarose Gel Isoelectric Focusing: Collaborative Study

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A method for fish species identification by agarose gel isoelectric focusing was collaboratively studied to determine if unknown samples could be identified by comparison of the unknown protein pattern with a photograph of protein patterns from authentic species. Fourteen collaborators were sent 19 or 20 unknown samples along with supplies and instructions for making pH 2.5-9.0 agarose isoelectric focusing gels. The unknown sarcoplasmic protein patterns were identified by comparison with a supplied 8 × 10 in. photograph of protein patterns from 18 authentic species. The 14 collaborators identified a total of 276 unknown samples with 84% accuracy. Eight of 14 collaborators averaged 90% or more correct. Two collaborators averaged 80-85% correct, while the remaining 4 collaborators averaged 50-69% correct. No one species was consistently misidentified; however, the method is not recommended for adoption in its present form.

Isoelectric focusing of sarcoplasmic proteins in polyacrylamide gels is an excellent method for the identification of fish species. A collaborative study of a polyacrylamide gel isoelectric focusing (PGIEF) method in which unknown species' patterns were identified by comparison with a photograph of protein patterns from authenticated species resulted in 93% of the unknown samples being correctly identified (1). All unknown species were correctly identified except for samples of monkfish (*Lophius americanus*). Monkfish was subsequently found to have a polymorphic protein pattern with 3 distinct variations detectable using the PGIEF method (2). PGIEF was adopted by the AOAC as an official final action method in October 1980, after one year at official first action with no further problems reported.

In view of the known toxicity of acrylamide, appropriate warnings were included in the method to protect potential users. However, because acrylamide is a potent cumulative neurotoxin, an alternative stabilizing medium was sought. The introduction by several manufacturers of a highly purified agarose suitable for use in isoelectric focusing led to the development of an alternative species identification method based on agarose gel isoelectric focusing (AGIEF) (3). Aside from the advantage of replacing the toxic acrylamide with nontoxic agarose, AGIEF has the added advantage of speed. The protein separation step can be completed in 30 min compared with 90 min for the PGIEF method, and the fixing, staining, and destaining steps are also much faster.

Fourteen collaborators were each sent 19 or 20 unknown frozen fish samples from 10 different species. Collaborators were also supplied with materials to produce pH 2.5-9.0 agarose gels, an 8 × 10 in. photograph of a pH 2.5-9.0 agarose gel showing protein patterns from 18 authenticated species and a copy of the proposed method. Because monkfish was found to have 3 distinct patterns, all 3 patterns were included on the "library gel" and each collaborator was given 2 of the 3 different monkfish types as unknown samples. The collaborators were instructed to analyze the unknown samples according to the supplied method and to identify the unknowns by comparing their protein patterns with the patterns shown on the photograph.

METHOD

Apparatus

(a) *Thin layer isoelectric focusing.*—LKB 2117 Multiphor for electrofocusing (LKB Instruments, Inc., 12221 Parklawn Dr, Rockville, MD 20852), or equivalent apparatus.

This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19-22, 1981 at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee C and was accepted by the Association (*J. Assoc. Off. Anal. Chem.* **65**, 373 (1982)).

Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service.

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(**b**) *Power supply.*—Constant power type capable of maintaining constant power up to 30 watts.

(**c**) *Constant temperature circulator.*—Capable of circulating water or antifreeze solution through cooling platform at 0–10°C.

(**d**) *Covered plastic or glass trays.*—To hold fixing, staining, and destaining solutions. Minimum size depends on size gel used. Some plastics may cause the stain to precipitate during the staining procedure.

(**e**) *Glass plates.*—125 × 260 × 3 mm for Multiphor or sized to fit apparatus in use.

(**f**) *Gel backing.*—GelBond, 0.2 mm thick, cut to size of desired gel (FMC Corp., Marine Colloids Division, Rockland, ME 04841).

(**g**) *Sample application foil.*—For agarose gels (Catalog No. 2117-206, LKB Instruments, Inc.).

(**h**) *Filter paper.*—S&S No. 577 (Schleicher & Schuell, 543 Washington St, Keene, NH 03431).

(**i**) *Drying apparatus.*—Infrared heat lamp or electric hair dryer.

Reagents

(**a**) *Fixing solution.*—Mix 150 mL methanol and 350 mL water. Add 17.25 g sulfosalicylic acid and 25 g trichloroacetic acid. Discard after one use.

(**b**) *Destaining solution.*—Mix 500 mL alcohol and 160 mL acetic acid. Dilute to 2 L with water.

(**c**) *Staining solution.*—Dissolve 1.15 g Coomassie Blue R-250 in 1 L destaining solution. Discard after one use.

(**d**) *Anode solution.*—0.1M H_3PO_4.

(**e**) *Cathode solution.*—0.1M NaOH.

(**f**) *Agarose.*—IsoGel Agarose (FMC Corp.).

(**g**) *Carrier ampholytes.*—LKB Ampholine, pH ranges 2.5–4, 5–8, and 3.5–10 (LKB Instruments, Inc.).

(**h**) *d-Sorbitol solution.*—20% (w/v)

Sample Preparation

Thaw frozen fish overnight in refrigerator or 1 h in cold water. Collect drip fluid that forms upon thawing. Remove suspended particles in drip fluid by centrifuging 5 min at ≥1000 g. Alternatively, filter drip fluid through Whatman No. 1 paper in refrigerator if centrifuge is not available. Centrifuged drip fluid may be stored at 4°C a maximum of 2 days before discarding.

Agarose Gel Preparation

The following directions produce a 125 × 260 × 1 mm thick gel suitable for use with Multiphor. Amounts can be adjusted to produce smaller or larger gels as needed.

Step 1—Add 0.24 g IsoGel agarose and 15 mL water to 50 mL screw-cap test tube. Melt agarose in 90–100°C water bath. Use immediately or let solidify at room temperature and store at 4°C. Discard when bacteria or mold growth is evident.

Step 2—Add 13.5 mL 20% (w/v) sorbitol (**h**) to melted agarose and mix.

Step 3—Add LKB Ampholine carrier ampholytes in the following proportions for pH 2.5–9.0 gradient: 0.9 mL pH 3.5–10; 0.3 mL pH 2.5–4; 0.3 mL pH 5–8. Mix well and let air bubbles rise while maintaining temperature at 80–90°C.

Step 4—Assemble gel mold. Place 5 mL water on center of glass plate. Roll piece of GelBond onto glass plate, spreading water evenly and avoiding air bubbles. Ensure that hydrophobic surface of GelBond faces down and hydrophilic surface faces up. Use clean test tube to roll GelBond flat and squeeze out excess water. Place 1 mm thick spacer and then top glass plate over GelBond. Clamp assembly together and preheat to 30–40°C in oven or by using infrared lamp or hair dryer.

Step 5—Pour agarose-sorbitol-Ampholine mixture into preheated 30 mL glass syringe fitted with 19 gauge needle. Pump hot mixture into preheated gel mold without trapping air bubbles.

Step 6—Let agarose solidify at room temperature and then transfer to 4°C refrigerator for ≥ 15 min before use. Gel may be stored at 4°C for 2 or 3 days maximum if open end of the gel mold is sealed with masking tape.

Step 7—Remove gel from mold by gently prying off top glass plate and lifting GelBond with adhering agarose gel free from bottom glass plate.

Step 8—Place GelBond on cooling platform over thin layer of water. Avoid air bubbles.

Step 9—Remove excess surface moisture from gel by gently rolling dry piece of S & S No. 577 filter paper onto gel surface without trapping air bubbles. Gently peel paper off gel when evenly saturated (ca 15 s). Do not dislodge agarose from GelBond. After blotting, agarose should have dull, even, matte surface. Any remaining glossy areas should be reblotted using same piece of filter paper. Incomplete surface moisture removal will cause adjacent samples to run together.

Step 10—Place sample application foil on agarose gel surface near cathode end of gel. Ensure that protrusions from each slit face gel.

Step 11—Pipet 1-2 μL sample into center of each slit. Allow time for sample to be absorbed, then remove foil carefully.

Step 12—Cut 2 electrode strips to length of gel. Saturate strips with 0.1M NaOH for cathode and 0.1M H_3PO_4 for anode strip. Place strips on gel surface under appropriate electrode.

Step 13—Place electrodes in position and connect electrical leads to power supply, observing proper polarity.

Step 14—Start flow of coolant through cooling platform.

Step 15—Turn on and set power supply to deliver constant power = 30 watts with limiting voltage = 1.5 kV and limiting current = 50 mA. Separation is complete after 30 min.

Step 16—Turn off power supply, remove and discard electrode strips.

Step 17—Place gel in fixing solution 10 min.

Step 18—Remove gel from fixing solution and roll piece of S & S No. 577 filter paper, wet with water, onto gel surface.

Step 19—Place gel filter paper down, on stack of dry paper towels. Place 500-1000 g weight on top of gel backing to ensure good contact with paper towels. Remove wet paper towels and replace with dry paper towels to speed dehydration. After 15-30 min, gel should have dried to "paper thin" layer. Peel filter paper off gel, wetting with water if paper sticks to gel.

Step 20—Dry gel fully using hair dryer or infrared lamp, until gel is dry to touch.

Step 21—Rinse dried gel 1 min in destaining solution.

Step 22—Stain gel 10 min with frequent agitation to prevent dye precipitation. If dye precipitates, discard solution and replace with fresh stain.

Step 23—Destain gel background with several 1 min washes of destaining solution, until background is clear.

Step 24—Dry destained gel using hair dryer or infrared lamp.

Step 25—Identify unknown samples by visual comparison of unknown pattern with protein patterns from authentic species.

Step 26—Dried gels may be written on and can be stapled into notebook as permanent record.

Results and Discussion

Table 1 summarizes the results obtained from the 14 collaborating laboratories. Eight of 14 collaborators identified the unknown samples with accuracy ranging between 90 and 100%. Two collaborators obtained between 80 and 85% accuracy, 2 collaborators obtained between 65 and 69% accuracy, and 2 collaborators obtained between 50 and 55% accuracy. Overall, 84.1% of the unknown samples were identified correctly. No one species was misidentified significantly more often than another, although only the monkfish (Type C) was correctly identified by all collaborators.

A number of problems were identified after visual examination of the gels submitted by each of the collaborators. Comments were also noted by several collaborators. In general, the collaborators who scored highest submitted gels which were similar in resolution to the library gel. Those collaborators who scored lowest submitted gels that differed in appearance from the library gel. Collaborators 6 and 7 reported deviation from the power supply conditions specified in the method, and this may have affected the resulting resolution of the proteins. Collaborator 7 also reported problems with the protein stain precipitating. Collaborator 4 submitted a gel with protein patterns that were stained a blue-green that differed from the dark blue typically seen with Coomassie Blue. It has recently been noted that the original manufacturers of Coomassie Blue R-250 no longer produce that product. Instead, several other manufacturers are producing "Coomassie Blue" products which may not be exactly the same. Different stains exhibit different binding responses to proteins, so this may be the cause of some of the protein pattern differences. Collaborator 3 did not submit a gel for examination, so no critical evaluation was possible.

Overall, the agarose gel isoelectric focusing method that was collaboratively studied showed more variability than the present AOAC method based on polyacrylamide gel isoelectric focusing (1). This fact was noted by most collaborators. Most collaborators also noted that they had one or more problems with the specified staining procedure. The identifications using the library gel were also a problem to most collaborators. Even the collaborators who did well complained that the large number of unknown samples combined with the large number of known patterns made the identifications difficult. Several collaborators felt that the collaborative study should have been conducted using both unknown and authentic samples run on the same gel because this is normally required in court cases. Nevertheless, the Associate Referee feels that the library gel comparisons are a more stringent test of the method reproducibility. At this point, identifications should not be based only on the library gel identifications. It would

Table 1. Summary of collaborative study results for fish species identification method based on agarose gel isoelectric focusing [a]

Species	Code	Collaborator														Correct/total
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Gray sole	A	C	C	C	C	C	X	X	--	C	C	C	C	C	X	10/13
Gray sole	V	C	C	C	C	C	X	X	C	C	C	C	C	C	X	11/14
Haddock	C	C	C	X	X	C	—	C	X	C	C	X	C	X	C	8/13
Haddock	R	C	C	X	C	C	C	—	X	C	C	C	C	X	C	10/13
Cod	E	C	C	X	C	C	X	C	X	C	C	C	C	C	X	10/14
Cod	P	C	C	X	C	C	X	X	C	C	C	C	C	C	X	10/14
Blackback	F	C	C	C	X	C	C	X	C	C	C	C	C	C	C	12/14
Blackback	T	C	C	C	X	C	C	X	C	C	C	C	C	C	C	12/14
Pollock	H	C	C	C	X	C	C	C	C	C	C	C	C	C	C	13/14
Pollock	L	C	C	C	X	C	C	C	C	C	C	C	C	C	C	13/14
Wolffish	I	C	C	C	C	C	C	C	C	C	C	C	C	C	C	14/14
Wolffish	S	C	C	C	X	C	C	C	C	C	C	C	C	C	C	13/14
Red hake	K	C	C	X	C	C	C	C	C	C	C	C	C	C	C	13/14
Red hake	O	C	C	X	C	C	C	X	C	C	C	C	C	C	C	12/14
Cusk	M	C	C	X	X	C	X	C	C	C	C	C	C	C	C	11/14
Cusk	W	C	C	X	C	C	X	C	C	C	C	C	C	C	C	12/14
Yellowtail	N	C	C	C	C	X	C	X	C	C	C	C	C	C	C	12/14
Yellowtail	X	C	C	C	C	C	C	X	C	C	C	C	C	C	C	13/14
Monkfish A	Z	C	C	X	C	—	—	X	C	—	C	—	C	C	—	7/9
Monkfish B	Y	C	—	X	—	C	C	—	C	C	—	C	—	C	C	8/9
Monkfish C	G	—	C	—	C	C	C	C	—	—	C	C	C	—	C	9/9
Correct/total		20/20	20/20	10/20	13/20	19/20	13/19	10/19	16/19	19/19	20/20	19/20	20/20	18/20	16/20	233/276

[a] C = correct identification; X = wrong identification; — = not supplied.

be more logical to use the library gel to pinpoint the suspected identity of an unknown sample and then to procure an authentic sample of that species to be run side-by-side with the unknown.

Aside from the above problems, the collaborators were unanimous in their agreement on the good points of the method. The relatively low cost, the lack of the polyacrylamide toxicity problem, and the great speed of the method were cited as being distinct advantages. Further study of the method was suggested.

Recommendations

The accuracy of this method, as determined by collaborative study, was not good enough for recommendation as official first action. The use of agarose gels in isoelectric focusing is a relatively new technique, and the technique is developing rapidly. Several different agarose-carrier ampholyte systems are now being marketed by several companies. During the next year, each of these systems should be evaluated to determine which are the most reproducible. A pre-blended carrier ampholyte preparation is also available, which should improve the reproducibility. A replacement for the discontinued Coomassie Blue R-250 stain is also needed to ensure reproducible staining results. The use of ultra-thin agarose gels, cast by capillary action, should be investigated to further improve reproducibility and to further reduce analysis time.

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METALS AND OTHER ELEMENTS

Gas-Liquid Chromatographic Screening Method for Determination of Methyl Mercury in Tuna and Swordfish

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A rapid screening method has been developed for determining methyl mercury in tuna and swordfish. Fish tissue is blended with acidic KBr solution to release methyl mercury, which is then extracted into methylene chloride. After cleanup by partitioning with cysteine, the methyl mercury is extracted into toluene and determined by gas-liquid chromatography. The proposed method compares favorably with the official AOAC atomic absorption method.

Mercury is routinely determined in tuna and swordfish by measuring total mercury content (1). However, it is well known that the bulk of mercury in fish is present as methyl mercury (2, 3). Because methyl mercury is highly toxic, from a regulatory and toxicological standpoint its determination is eminently more important than that of metallic mercury.

Methods presently in use for the direct determination of methyl mercury all have some undesirable or difficult features, which have been documented previously (2, 4–9). In addition, most of the methods use benzene, a known carcinogen (10), as the primary solvent for extraction.

In an effort to obviate the use of benzene, to mitigate undesirable features of existing methods, and to provide an expeditious screening method for routine determination of methyl mercury in fish, the method described below was developed. It is essentially a modification of the Newsome method (6), as follows: Methyl mercury is released from fish tissue by blending with acidic KBr solution, and is then extracted into methylene chloride and partitioned with cysteine solution to provide cleanup; the cysteine is acidified with HBr, and methyl mercury is extracted into toluene and determined by electron capture (EC) gas-liquid chromatography (GLC).

METHOD

Reagents and Apparatus

(a) *Solvents.*—GLC grade methylene chloride, acetone, petroleum ether, and toluene.

(b) *Acidic potassium bromide solution.*—Dissolve 200 g KBr in ca 500 mL water. Add 100 mL concentrated H_2SO_4, cool, and dilute to 1 L with water.

(c) *Cysteine acetate solution.*—Dissolve 1.0 g cysteine hydrochloride monohydrate, 4.0 g sodium acetate trihydrate, and 12.5 g anhydrous sodium sulfate in water and dilute to 100 mL. Prepare fresh every 2 days.

(d) *Hydrobromic acid.*—48%, ACS grade.

(e) *Methyl mercury chloride standard solutions.*—(1) *Stock solution.*—100 μg/mL in acetone. (2) *Intermediate solution.*—3 μg/mL in acetone. Dilute 3.0 mL stock solution to 100 mL with acetone. (3) *Working solution.*—0.12 μg/mL in toluene. Dilute 1.0 mL intermediate solution to 25.0 mL with toluene. Inject ca 5 μL.

(f) *Gas chromatograph.*—Tracor 560, or equivalent, equipped with EC ^{63}Ni detector and 4 ft × 2 mm glass column. Column packing: 10% DEGS–1% H_3PO_4 on 80–100 mesh Chromosorb WAW; evacuated 30 min with helium; conditioned 24 h at 230°C; and "primed" with mercuric chloride by injecting 100 μL of a 1 mg/mL solution in acetone at 115°C 24 h before use. Operating conditions: temperatures—column, 115°C; detector, 300°C; injection port, 180°C; purge rate, 60 mL argon–methane/min; flow, 20 mL argon–methane/min; saturation current, 8 × 10^{-9} A; recorder attenuation, 5.

Preparation of Sample

(a) *Canned fish.*—Drain fish and composite by chopping in Hobart chopper.

(b) *Fresh or frozen fish.*—If frozen, allow to thaw; then composite edible portions as in (a).

Determination

Accurately weigh 10.0 g prepared fish sample (5.0 g or less for swordfish), and transfer to micro-blender or homogenizer. Add 100 mL acidic KBr solution and blend at moderate speed ca 10 min. Filter with suction through Buchner funnel fitted with sharkskin filter.

Transfer 50.0 mL filtrate to 500 mL separatory funnel. Extract filtrate with three 100 mL portions of methylene chloride, combining meth-

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ylene chloride extracts in another 500 mL separatory funnel. Add 20.0 mL cysteine acetate solution, shake vigorously ca 2 min, and discard methylene chloride layer.

Wash cysteine acetate layer with 50 mL petroleum ether, let layers separate, and transfer cysteine acetate layer to 125 mL separatory funnel. Acidify cysteine acetate layer with 2 mL 48% KBr. Add exactly 10.0 mL toluene and shake vigorously ca 2 min.

Collect toluene layer and inject ca 5 μL into gas chromatograph for methyl mercury determination.

Results and Discussion

The proposed method is applicable to occasional analyses or to routine multiple analyses. Time for a single determination is about 1 h, and 8–12 samples per day can easily be accommodated.

In the initial experiments, HCl was used instead of H_2SO_4 in the preparation of acidic KBr. However, some emulsions were encountered in the methylene chloride extraction step. The switch to H_2SO_4 moderated this effect considerably.

The gas chromatograph columns were conditioned 24 h at 230°C and primed with mercuric chloride by injecting 100 μL of a 1 mg/mL solution in acetone at 115°C, 1 day before use. This resulted in a stable column which kept the sensitivity and retention time of methyl mercury fairly constant for 2–3 days.

To test recovery of methyl mercury, a solution of 1.4 μg methyl mercuric chloride/mL in acidic KBr was prepared. Replicate 2.0 mL aliquots were carried through the proposed method. Based on a 10 g sample weight, this is equivalent to 0.28 ppm methyl mercuric chloride. The average recovery for 12 determinations is 95.6% with a standard deviation of ±1.64.

To test recoveries from a real sample matrix, 8 analyses were performed on NBS Research Material 50 (Albacore Tuna). The average amount of methyl mercury, calculated as mercury, was 0.89 ppm by the proposed method. NBS gives the noncertified value as 0.95 ± 0.1 ppm, and they suggest that 80–90% of the mercury content is methyl mercury. Repeated atomic absorption analyses of the NBS Research Material in our laboratory gave an average mercury content of 0.86 ppm.

Table 1 compares the proposed method with the official atomic absorption method (1). The analyses were performed over a 2 month period and included analyses of canned, frozen, and decomposed fish. Some gas chromatographic interferences (probably putrefaction products of the fish) were noted in fish samples that had begun to decompose, but the overall results obtained by the proposed method compare quite favorably with those by the official method.

Table 1. Comparative results of mercury determination by AOAC official method and proposed method

Product	Hg found, ppm: AOAC 25.110–25.114	Hg found, ppm: Proposed method
Tuna (canned)	0.17	0.16
	0.20	0.19
	0.07	0.09
	0.04	0.07
	0.05	0.08
	0.23	0.23
	0.24	0.21
	0.07	0.10
	0.11	0.14
	0.47	0.42, 0.39
	0.18	0.18, 0.18
	0.15	0.15, 0.15
	0.69	0.60, 0.62
Swordfish (frozen)	0.57	0.60[a]
Swordfish (decomposed)	1.15, 1.19	1.11[a]
Swordfish (frozen)	0.64	0.63
	0.58	0.48
	0.90	0.78
	0.28	0.32

[a] Some interferences noted on gas chromatograms.

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Optimum Conditions for Hydride Generation of Selenium and Its Determination by Atomic Absorption Spectrophotometry

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Instrumental parameters and other variables were studied to establish the optimum indicators for determination of selenium by atomic absorption spectrophotometry using the hydride generation procedure with cold-trapping. Optimum conditions were established by consideration of the following variables: volume of reaction solution, acid concentration in the reaction mixture, lapse of time after last addition of borohydride, method of addition of borohydride, carrier gas flow rates, position of source beam in the flame cell, oxidation state of selenium, and effect of drying the cold trap between successive determinations. This latter procedure greatly improved the reproducibility of the technique (from ±28% to ±2%. The limit of detection was about 2 ng selenium.

Extensive literature exists on the hydride generation method for determining selenium by atomic absorption spectrophotometry (1–8). The most common techniques (1–5) involve reduction of selenium by sodium borohydride and introducing the hydride by means of a carrier gas (nitrogen) directly into an air-entrained nitrogen–hydrogen flame. Siemer and Koteel (2) studied the effect of freezing the hydride in liquid nitrogen by use of the method of Knudsen and Christian (7) and reported better efficiency of transfer of the hydride compared with the direct method (33% compared with 14%). A disadvantage of the freezing-out technique, however, was the nearly 3-fold poorer precision.

The relatively poor precision of hydride generation methods for selenium determinations by atomic absorption spectrophotometry was further demonstrated by Ihnat and Miller (8) in an extensive intra- and interlaboratory study of selenium levels in 13 types of foodstuffs containing 31–3625 ng selenium/g. Relative standard deviations (RSD) between laboratories (interlaboratory) ranged from 14% at the 2538 ng/g level to 275% at the 31 ng/g level. Intralaboratory studies (between digests) gave RSD values of 7.4 and 99%, respectively.

We recently studied parameters affecting hydride generation of arsenic and its determination by atomic absorption spectrophotometry (9) and have now carried out a similar study for the determination of selenium with a view to improving the precision of the method. The results of this work are presented in this paper.

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Experimental

Reagents

Analytical grade reagents were used throughout. Fresh selenium(IV) or selenium(VI) solutions were prepared by weighing the appropriate amount of sodium selenite or selenatel and dissolving it in deionized water. Sodium borohydride solutions were prepared daily using deionized water made alkaline by the addition of a few pellets of potassium hydroxide. To prevent condensation of volatile acidic vapors in the cold trap, sulfuric acid was used to acidify the sample before injection of the reducing agent.

Apparatus

The apparatus is illustrated in Figure 1 and is identical to that used by us earlier on arsenic (9). Selenium hydride is generated in the reaction vessel and carried by a stream of nitrogen to the condenser, which is immersed in liquid air. The condenser is connected to the mixing chamber and burner unit of a Varian Techtron AA-5 atomic absorption spectrophotometer. The selenium resonance line at 196.0 nm was used. The source, a Cathodeon hollow cathode lamp, was operated at 6 mA. Absorption signals were recorded on a Rikadenki B-161 chart recorder operated at 10 cm/min. The attenuator setting was generally either 2 or 5 mV. A nitrogen-hydrogen-entrained air flame was used for production of ground-state atoms.

Original Basic Operating Conditions

The following basic operating procedure was used throughout and was only modified when a specific parameter (e.g., acid concentration or total volume) was varied purposely. Deionized water (40 mL) and 10 mL of 3M sulfuric acid were placed in the reaction flask. Selenium(IV) solutions were introduced by an Eppendorf pipet.

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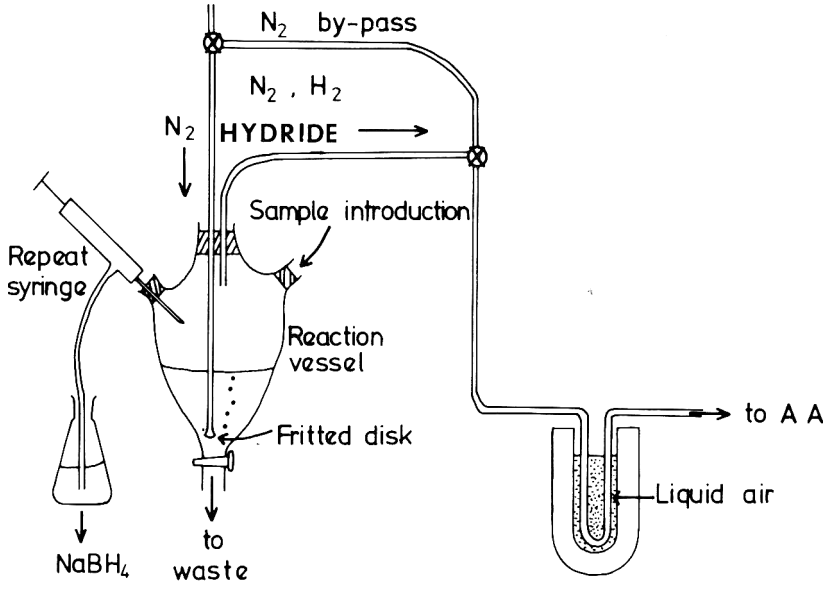

Figure 1. Apparatus for generation of selenium hydride.

The condenser consisted of a 10 mm (id) borosilicate tube immersed in liquid air to a depth of 150 mm. A stream of nitrogen was passed through the reaction solution. When the condenser had cooled, sodium borohydride was introduced, 2 mL at a time, using a repeating syringe. The hydride was carried by the nitrogen stream to the condenser. After sufficient reaction time, the nitrogen stream was rerouted to bypass the reaction vessel, and the condenser was placed in an ice-water bath where the hydride rapidly volatilized and was carried into the flame. The reaction flask was washed before the next sample was added.

Results and Discussion

Amount of Borohydride Used

In this experiment, 1 μg selenium(IV) was reacted with successive 2 mL aliquots of 3% sodium borohydride solution injected at 30 s intervals. The first addition produced a signal about 25% of the final limiting value, which was reached after 4 mL had been added. An insurance against incomplete conversion of selenium to the hydride, however, a total of 8 mL of reagent was added in 4 increments of 2 mL.

Total Reaction Volume

To evaluate dependence of the absorption signal on the initial reaction volume, a series of solutions were prepared containing a total volume between 10 and 90 mL and containing 1 μg selenium(IV) as well as a constant concentration (0.6 M) of sulfuric acid.

The results are shown in Figure 2. It will be noted that the total signal decreases slightly (by about 10% as the reaction volume changes from 10 to 90 mL. It would appear that the efficiency of removal of selenium hydride is not greatly affected by the reaction volume. The reaction volume of 50 mL recommended in the standard procedure represents an effective compromise between sensitivity and reproducibility because at lower reaction volumes the rate of change of signal is slightly greater than for higher volumes. This means that the gain in sensitivity by use of lower reaction volumes would be at the expense of poorer reproducibility.

Effect of Acidity

A series of 6 solutions were prepared containing 1 μg selenium(IV) in 10M, 8M, 6M, 4M, 2M, and 1M sulfuric acid. The resultant signals were approximately identical, and it appeared that release of selenium hydride was independent of the acid concentration, at least in this range of values.

Rate of Stripping of Selenium Hydride from the Cold Trap

Eight replicate solutions containing 1 μg selenium were treated with borohydride, and the

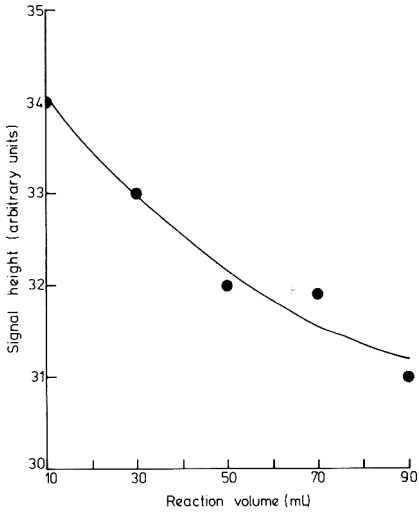

Figure 2. Atomic absorption of selenium as function of total reaction volume.

hydride was collected in a cold trap. The carrier gas was fumed through the system for periods of 25–200 s after the last addition of reagent. The hydride was then volatilized and poured into the flame in the usual manner. The results of this experiment are shown in Figure 3.

The signal increased gradually for the first 120 s, probably because of slow removal of the last traces of hydride from the solution. Thereafter, there was a slow reduction of signal as the solid hydride was stripped from the U-tube. The loss, however, was slight and amounted to only about 5% of the maximum value (at 200 s).

Carrier Gas Flow Rates

The result of altering the carrier gas flow rates for replicate solutions containing 1 μg selenium(IV) is shown in Figure 4. The pattern was very similar to that previously obtained for arsenic (9) with a maximum signal at a flow rate of around 3 L/min. At higher flow rates, the selenium hydride apparently does not have time to condense completely, whereas at low flow rates the signal is reduced because of greater diffusion of the hydride into the carrier gas.

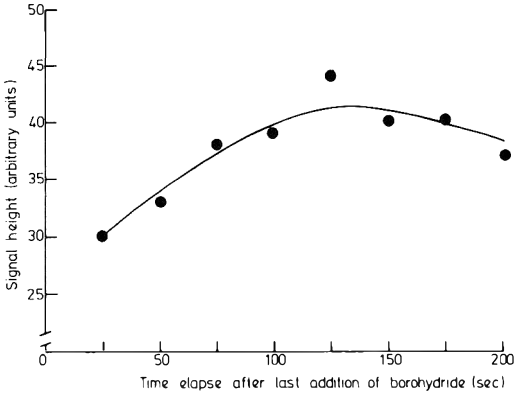

Figure 3. Atomic absorption of selenium as function of time elapse since last addition of borohydride.

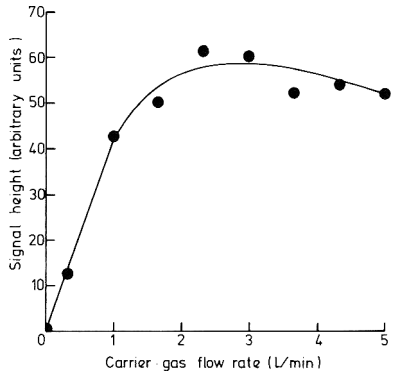

Figure 4. Atomic absorption of selenium as function of carrier gas (nitrogen) flow rate.

Varying Gas Mixtures in the Flame Cell

The effect of varying ratios of flow rates of nitrogen and hydrogen supplied to the flame cell was investigated by preparing a number of replicate solutions with 1 μg selenium(IV). Hydride generation and flame analysis was carried out with one set of samples in which the nitrogen was kept constant at 5 scale units and the hydrogen was varied from 1 to 10 scale units. A second set was treated in which the hydrogen was kept constant at 5 units and the nitrogen was varied from 1 to 10. The constant flow rates for nitrogen and hydrogen were not chosen arbitrarily but were a compromise in the middle of the range of flow rates of either gas for which it is possible to sustain a stable flame. It must be mentioned that the flow rate units were those of the scale divisions of the AA-5 instrument and overstate time flow rates (mL/min) by around 150%. The data are shown in Figure 5. It seems that the gas mixture has a great influence on the signal and that flow rates of either gas should preferably be in the range of 4–7 scale units (i.e., about 3–5 L/min).

Position of the Source Beam in the Flame

The influence of the height of the source beam within the flame was investigated, and the results are shown in Figure 6. The signal was an

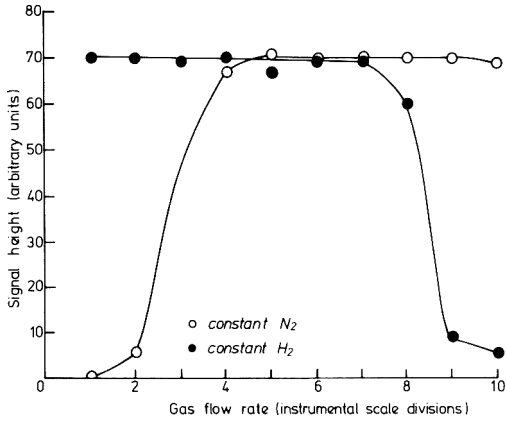

Figure 5. Atomic absorption of selenium as function of gas composition of flame cell.

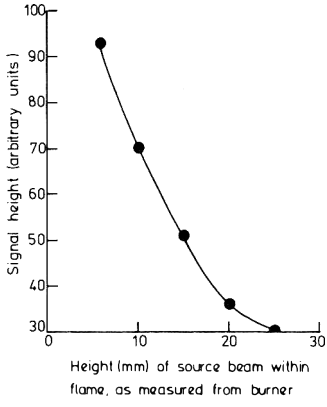

Figure 6. Atomic absorption of selenium as function of position of source beam within flame.

inverse function of the height of the source beam within the flame. It is clear that this beam must be kept as close as possible to the slit of the burner. Even a few millimeters difference can be crucial, particularly if maximum sensitivity is required.

Effect of the Oxidation State of Selenium

Experiments were carried out using solutions of selenium in both the hexavalent and tetravalent states. The oxidation state was very important in these experiments because a solution of selenium(IV) that gave a signal of 38 units compared with a value of only 3 units for the same quantity of selenium(VI). Attempts to reduce the selenium(VI) to selenium(IV) by additional quantities of borohydride were only partially successful and, in any case, involved use of an inordinate amount of expensive reagent. When the solution of selenium(VI) was reduced by boiling for 10 min in 4M hydrochloric acid, exactly the same signal as for selenium(IV) was obtained when borohydride was added in the standard manner. Pre-reduction of selenium, therefore, is essential when using the hydride generation technique.

Reproducibility and Limit of Detection

The purpose of the work was to examine the optimum instrumental conditions for the determination of selenium; therefore no attempt was made to assess the accuracy of the procedure by analyzing standard rocks because this would have brought in the question of error inherent in decomposition procedures. For the same reason, selenium levels in reagent blanks were not determined.

The limit of detection of the procedure (signal-to-noise ratio of 2.0) was of the order of 2.0 ng selenium.

In the earlier part of this investigation, it was realized that reproducibility was very poor and the relative standard deviation (20 replicates) for 1 μg quantities was as high as 28%. A dramatic improvement was effected by washing the tube with acetone and drying it with compressed air after each determination. The result of this procedure was a marked improvement in reproducibility (2.0%). Where necessary, all previous experiments were repeated with acetone-washed tubes. This washing is a very important prerequisite for reproducible work, and its importance cannot be overemphasized.

Optimum Operating Conditions

As a result of the above studies, we have now established the following optimum operating conditions for the determination of selenium: (*1*) volume of reaction solution, 50 mL; (*2*) acid concentration, 0.60M sulfuric acid; (*3*) lapse of time after last addition of borohydride, 120 s; (*4*) addition of borohydride, 4 increments of 2 mL at intervals of 30 s; (*5*) carrier gas flow rate, 3 L/min; gas mixture in flame cell, 3–5 L/min for both hydrogen and nitrogen; (*6*) position of source beam in flame, 1 or 2 mm above the burner; (*7*) oxidation state of selenium, IV (pre-reduce if necessary by boiling for 10 min in 4M hydrochloric acid); (*8*) dry U-tube between each sample by washing with acetone and evaporating the acetone with a stream of compressed air; (*9*) analytical line, 196.0 nm (this line was not deliberately chosen as a result of our studies and may not necessarily have given the best signal-to-noise ratio).

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NUTRITION

High Performance Liquid Chromatographic Determination of *Bifidobacterium bifidum* Growth Factors in Human Milk

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An isocratic high performance liquid chromatography (HPLC) method has been developed for the determination of *Bifidobacterium bifidum* growth factors in human milk. The method involves the gradual addition of 3 volumes of ethanol to the milk sample, filtration, and analysis of the growth factors in the filtrate by HPLC. The HPLC system consisted of a carbohydrate analysis column, a water-acetonitrile (70 + 30) solvent system, a flow rate of 1.0 mL/min, and a refractive index detector. The method is simpler and requires less time than the present microbiological method. Moreover, it revealed for the first time the presence of 2 separable growth factors in all human milk samples tested. The HPLC method developed is sensitive and can be used to monitor the type and the amount of growth factors in mothers' milk during lactation.

Human milk contains specific factors that promote the growth of *Bifidobacterium bifidum* in the intestines of breast-fed infants (1-5). *B. bifidum* comprised more than 95% of the intestinal microflora of breast-fed infants and only 30-40% of the flora of formula-fed infants (6). The predominance of the acid-producing *B. bifidum* lowers the pH in the intestines of breast-fed infants and the induced acidity prevents the growth of harmful bacteria (7-10). The growth factors in human milk are therefore related to the lower mortality rate of breast-fed infants and their enhanced resistance to enteric and other infections during the critical months of infancy (1, 7-10).

Gyorgy et al. (11) tested the activity of human milk and milk of other species with *B. bifidum* var. *pennsylvanicus* and reported that human colostrum had the highest activity and human milk had 30-75 times the activity of milks from ruminants, including cows' milk. The growth factors in human milk were heat-stable and were not destroyed or altered by autoclaving (2). Gyorgy et al. (12) separated the growth factors in human milk into a dialyzable fraction representing from 40 to 75% of the total activity, and a relatively nondialyzable fraction of 20-60%. Rose et al. (13) reported that the growth factors in human milk consist of a group of nitrogen-containing saccharides.

The only quantitative method used to determine the growth factors in human milk at the present time is the microbiological method developed by Gyorgy (1). The method involves tedious media preparations and requires an incubation time of 40 h. The main objective of this study was to develop a more convenient and accurate high performance liquid chromatographic (HPLC) method for the determination of the growth factors in human milk.

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Experimental

Materials

A freeze-dried culture of *B. bifidum* var. *pennsylvanicus* (No. 11863) was obtained from the American Type Culture Collection, Rockville, MD. Acetonitrile, HPLC grade, was purchased from Waters Associates, Milford, MA. Chemicals used in preparing the growth media and other organic solvents were analytical reagent grade.

Apparatus

A Waters Associates liquid chromatograph used for HPLC analysis was equipped with Model 6000A pump, Model U6K injector, Model 401 differential refractometer, and a data module. A Labconco Model 3 freeze-drier (Labconco, Kansas City, MO) was also used.

Samples

Human milk samples were obtained from 16 healthy lactating mothers at 2-28 weeks postpartum. The lactating mothers were given polyethylene breast pumps (Kaneson, Inc., Los Angeles, CA) and were instructed to express approximately 0.5 oz of milk from the same breast before and after infant feeding. The samples were kept refrigerated until skimmed at 15 000 × g for 10 min. Equal volumes from the skimmed human milk samples were added to-

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gether as a pooled human milk sample. The pooled and the other skimmed human milk samples were cryogenically frozen and stored at −18°C until use.

Isolation of B. bifidum Growth Factors

Three 5 mL aliquots of the pooled skimmed human milk sample were used to compare the recovery of the growth factors from human milk by dialysis and by ethanol addition to the human milk. The first aliquot was pipetted into dialysis tubing and dialyzed at 4°C against 20 volumes of water for 24 h; the water was changed every 8 h. The dialysates were combined, freeze-dried, and reconstituted with water to a final volume of 1 mL. Ethanol (15 mL) was added gradually to the second aliquot with continuous gentle stirring and the mixture was stirred for an additional 15 min. The mixture was then filtered through Whatman No. 1 paper, and the filtrate was reduced in volume to 1 mL under vacuum. Equal volumes of the untreated third aliquot, human milk dialysate, and human milk filtrate were tested for growth factors by the microbiological assay.

HPLC Analysis

The HPLC conditions used were as follows: 30 cm × 3.9 mm carbohydrate analysis column fitted with C_{18} Porasil B guard column (Waters Associates); water–acetonitrile (70 + 30) solvent system; flow rate 1.0 mL/min; differential refractometer detection at 8× attenuation.

Appropriate volumes of either human milk dialysates or human milk filtrates (both samples had a pH value of 6.8) were injected and the solvent fractions corresponding to all peaks and zones between peaks were collected separately in test tubes. Fractions were evaporated to dryness under vacuum and redissolved in small volumes of water. The activity of all fractions was tested by the microbiological assay. The retention times of the peaks with growth activity were determined and the area of these peaks was obtained in data module units and in sq. mm by triangulation.

Microbiological Assay

The growth factors in the various human milk samples and fractions were determined by using a microbiological assay adapted from Gyorgy (1). A stock culture of *B. bifidum* var. *pennsylvanicus* was prepared in *Lactobacillus bificus* medium (LBM) (1, 14). Appropriate volumes of the samples to be tested were pipetted into tubes containing 10 mL LBM and autoclaved 15 min at

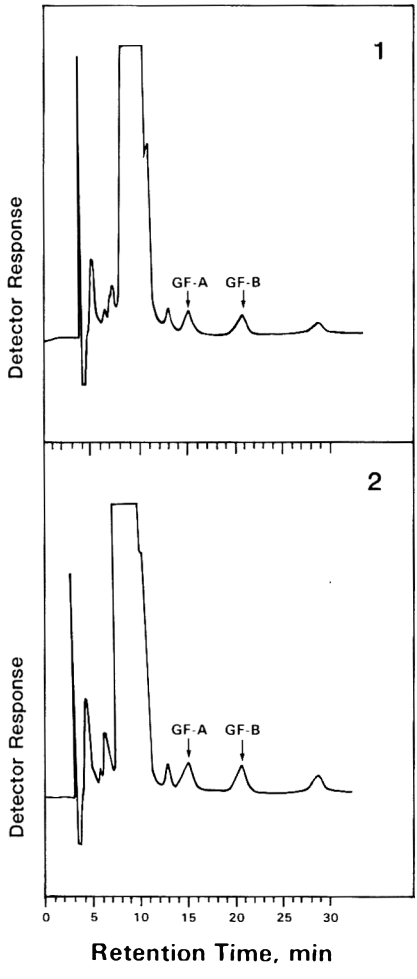

Figure 1. HPLC analysis of (1) skimmed human milk water dialysate and (2) skimmed human milk ethanol filtrate. Samples injected were equivalent to 25 μL skimmed human milk.

121°C. Sterile tubes were inoculated with the stock culture (1% v/v) and incubated 40 h at 35°C. Tubes were then centrifuged and supernates were titrated with 0.1N NaOH in the presence of phenolphthalein indicator. The volume of 0.1N NaOH required for titration was taken as a measure of the acid produced during the bacteria growth, which reflects the amount of growth factors present in the sample tested.

Results

Isolation of B. bifidum Growth Factors

The percent activity of growth factors isolated from skimmed human milk by dialysis and by ethanol filtrates were 69.1 ± 2.23 and 85 ± 2.06, respectively, for 3 tests (skimmed human milk =

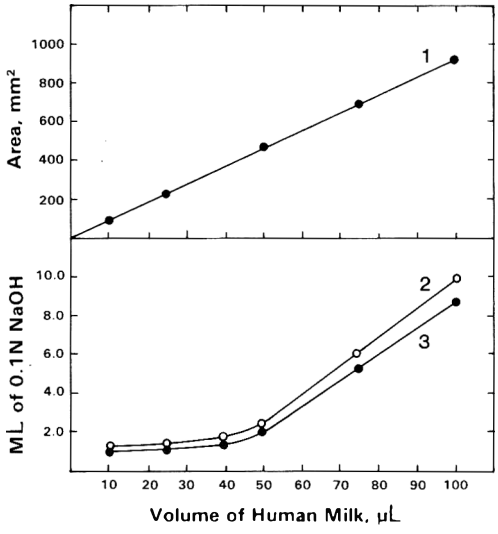

Figure 2. Calibration curves for growth factors in skimmed human milk ethanol filtrate (curve 1 from HPLC and curve 3 by microbiological assay) and skimmed human milk (curve 2 from microbiological assay). Each point represents average of 2 determinations.

100%). Higher quantities of growth factors were recovered in the ethanol filtrate, so the ethanol addition method was chosen over dialysis for further analysis.

HPLC Analysis of Pooled Human Milk Dialysate and Ethanol Filtrate

Chromatograms of equal volumes of pooled human milk dialysate and filtrate did not show any significant qualitative differences, as shown in Figure 1. Both chromatograms had the same number of peaks with similar retention times. In both samples, only 2 fractions corresponding to peaks with retention times of 15.3 and 20.7 min showed growth activity in the microbiological assay. None of the other fractions collected showed any appreciable growth activity. These results indicate the presence of 2 separable growth factors in human milk. These 2 factors are designated growth factor A (GF-A) and growth factor B (GF-B). The 2 factors present in the pooled skimmed human milk sample had similar peak areas and specific growth activity.

Sensitivity Study

To compare the sensitivity of the developed HPLC method with that of the microbiological assay, volumes of the pooled skimmed human milk sample (10, 25, 40, 50, 75, and 100 µL) were tested with the microbiological assay and the

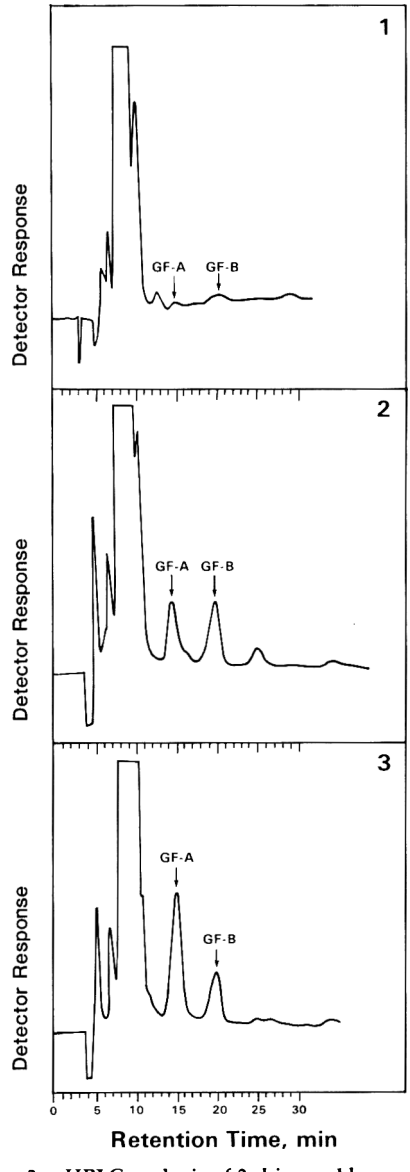

Figure 3. HPLC analysis of 3 skimmed human milk samples. Injections were equivalent to 50 µL skimmed human milk.

HPLC method. Volumes of 0.1N NaOH used in titration (microbiological assay) and the total area in sq. mm of the growth factors peaks (HPLC) were plotted against volumes of human milk sample analyzed. The results are shown in Figure 2.

Variation of the Growth Factors in Human Milk Samples

Results obtained from testing 3 human milk samples with marked differences in their growth

Table 1. Variation of *B. bifidum* growth factors in human milk samples

Sample	Peak area,[a] sq. mm GF-A[c]	GF-B[c]	(GF-A + GF-B)	0.1N NaOH,[b] mL
1	30	62	92	2.0
2	253	310	563	7.9
3	516	200	716	10.2

[a] Average of 2 HPLC runs; each injection volume was equivalent to 50 μL skimmed human milk.

[b] Average of 2 microbiological assays of skimmed human milk (100 μL).

[c] Growth factors A and B peaks (see Figure 3).

factors content are shown in Figure 3 and Table 1.

Discussion

Gyorgy et al. (12) reported that only 54% of the growth factor in human milk was recovered after 24 h of dialysis and that the recovery increased to 69% after dialyzing for 72 h. Under the dialysis conditions used in this study, the average percent recovery of the growth factor was 69.1 ± 2.33 after 24 h of dialysis, and it did not increase significantly when the same samples were dialyzed for 72 h. This may be due to the different dialysis conditions used by Gyorgy et al. (12). They dialyzed human milk samples against only 10 volumes of water without frequent changes of water during the dialysis. The addition of 3 volumes of ethanol to skimmed human milk resulted in an average percent recovery of 85.7 ± 2.06. When equal volumes of skimmed human milk dialysate and skimmed human milk ethanol filtrate were analyzed by HPLC, no qualitative differences in their chromatograms were observed (Figure 1). Both preparations yielded 2 growth factor peaks with identical retention times. The area of each growth factor peak in the filtrate was always greater than that of the equivalent peak in the dialysate. These results indicated that the addition of 3 volumes of ethanol did not alter the nature of the growth factors in skimmed human milk and that both growth factors were more extractable with approximately 75% aqueous ethanol than in water alone. Since the ethanol addition method has the advantages of greater percent recovery, shorter analysis time, and greater convenience, it was preferred to the dialysis method for isolating the growth factors from skimmed human milk for HPLC analysis.

Several solvent systems of different compositions of methanol–water, methanol–water–acetic acid, and water–acetonitrile were tried for HPLC analysis of the growth factors. Only water–acetonitrile (70 + 30) solvent system provided the best separation of the growth factors. When solvent fractions corresponding to each of the 2 growth factors peaks were collected and further analyzed by HPLC using several solvent systems of different strengths, only one peak was obtained in all runs. It was therefore concluded that only one compound was most likely present under each of the growth factors peaks. These results revealed for the first time the presence of 2 separable growth factors in skimmed human milk and that what is referred to in the literature as the *B. bifidum* growth factor in human milk is actually 2 factors. Both factors are dialyzable in water and are soluble in approximately 75% aqueous ethanol. These findings may be useful in future studies involving the identification of these growth factors in human milk.

Excellent linear responses over wide concentrations of the growth factors were obtained by the developed HPLC method (Figure 2). The HPLC method yielded better linear responses than the microbiological assay at volumes lower than 50 μL of skimmed human milk (Figure 2). For example, when the volume of the sample analyzed for growth factors increased from 10 to 25 μL (150% volume increase), the increase in the area of both growth factors (HPLC) was 144.6%, whereas the increase in volume of 0.1N NaOH required for titration (microbiological assay) was only 20%. The developed HPLC method was also sensitive; the growth factors in as little as 10 μL of skimmed human milk could be quantitated reliably.

Some of the human milk samples showed remarkable differences in their growth factors content as shown in Table 1 and Figure 3. Even though a good correlation existed between both methods in total growth factor activity, the HPLC method revealed differences in the milk samples content of each of the growth factors. The activity of GF-A in the milk samples from mothers 1 and 2 was 32.6 and 44.9%, respectively, of the total activity whereas it was 72.1% in the milk sample from mother 3. Such valuable information cannot be obtained by the microbiological assay. The developed HPLC method can therefore be used as a convenient screening test for determining content and make-up of the growth factors in milk samples from lactating mothers who breast-feed their infants. Supplementation of human milk or infant formulas with the deficient growth factor(s) may be needed for better health and survivability of infants. Studies on the isolation of large quan-

tities of both growth factors from whey for supplementation purposes are now under way in our laboratories.

Acknowledgments

The authors thank Jim Welty and Maura Carney for their technical assistance.

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DRUGS

Spectrophotometric Determination of Aminacrine Hydrochloride in Creams, Jellies, and Suppositories

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A visible spectrophotometric method has been developed for the quantitation of aminacrine hydrochloride in creams, jellies, and suppositories. Aminacrine hydrochloride was extracted into acidic ethanol and its visible spectrum was recorded. The amount present was calculated by determining the net absorbance between the absorbance maximum at about 402 nm and one-half the sum of the absorbances of the minima at about 389 and 412 nm. Aminacrine and a trace contaminant, 9(10*H*)-acridone, were independently identified by different thin layer chromatographic systems.

Because of its antiseptic properties, aminacrine hydrochloride, 9-aminoacridine hydrochloride (1), is incorporated into pharmaceutical preparations used to treat vaginal infections. The antiseptic activity of this acridine derivative is attributed to its ability to form nonionizing or feebly ionizing complexes with the acidic groups of nucleoproteins in the bacteria cytoskeleton. Acridine derivatives possess a broad bactericidal spectrum; they are effective against anaerobes, species of the proteus group, and other Gram-positive and Gram-negative bacilli (2–4).

There are no official methods for the determination of aminacrine hydrochloride in combination with other drugs. A fluorometric method for determining aminacrine hydrochloride had official first action status in the 12th edition of *Official Methods of Analysis* (5) but was deleted from the 13th edition (6) when the Associate Referee found that the ratio of the 2 peaks in the emission spectrum varied with concentration because the excitation and emission wavelengths overlap. Although aminacrine hydrochloride has a quantum efficiency close to 1, this reabsorption of emitted radiation precludes quantitation by fluorescence measurement.

An alternative procedure has been reported in the USP XX (7). This method uses a visible spectrophotometric assay only for aminacrine-4-hexylresorcinol (1 + 1) compound (acrisorcin) in a cream. The complex is quantitated by measuring the aminacrine hydrochloride absorbance at 402 nm. The method reported in this paper is a modification and extension of the USP monograph in that it employs a different extraction procedure and includes not only other dosage forms but also other drugs in combination with aminacrine hydrochloride. The sample is dissolved in acidic alcohol and the absorption spectrum is determined for an aliquot of the sample. No further cleanup to remove either excipients or other active drug ingredients is required.

Both aminacrine hydrochloride and its trace contaminant, 9(10*H*)-acridone, were identified by thin layer chromatography (TLC). For aminacrine hydrochloride, the alcohol extract from the assay was spotted on a silica gel plate, developed with ethyl acetate–methanol–concentrated ammonium hydroxide (17 + 3 + 2), and visualized with ultraviolet (UV) light. The presence of acridone was detected and confirmed using silica gel plates with either benzene–methanol (95 + 5) or methylene chloride–diethylamine (2 + 1) developing solvents along with UV light detection. Both aminacrine hydrochloride and acridone exhibit bright blue fluorescence under shortwave and longwave UV light. Aminacrine is resolved from the other drugs found in its formulations by the proposed TLC identification test.

METHOD

Apparatus and Reagents

(**a**) *Recording spectrophotometer.*—Hitachi Model 110 supplied with 1 cm quartz cells or spectrophotometer suitable for measurement in the 500–300 nm range.

(**b**) *Acidic ethanol.*—Concentrated HCl in ethanol (1 + 99).

(c) *TLC plates.*—20 × 20 cm glass, precoated with 250 μm layer of silica gel with or without fluorescent indicator.

(**d**) *TLC developing solvent.*—Ethyl acetate–methanol–concentrated NH_4OH (17 + 3 + 2).

[1] Associate Referee for Aminacrine and Quinacrine Hydrochloride.

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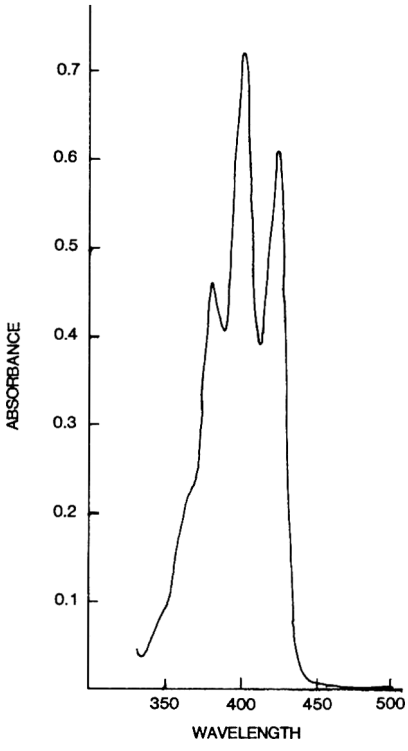

Figure 1. Visible spectrum of 0.0156 mg aminacrine hydrochloride/mL acidic ethanol.

(e) *Standard.*—Dissolve ca 25 mg aminacrine hydrochloride (Sigma Chemical Co., No. A-1135, or equivalent) in 100.0 mL acidic ethanol and dilute with acidic ethanol to 0.015 mg/mL for creams, jellies, and 6 mg suppositories and to 0.0175 mg/mL for 12 and 14 mg suppositories.

Sample Preparation

Creams and jellies.—Accurately weigh sample equivalent to 1.5 mg aminacrine hydrochloride into 150 mL beaker. Add ca 40 mL acidic ethanol and heat on steam bath 10 min with occasional stirring. Cool and quantitatively transfer to 100 mL volumetric flask and dilute to volume with acidic ethanol. Filter solution, discarding first 10 mL filtrate.

Suppositories.—Molded.—Determine average individual weight and composite 5 suppositories by heating in 70°C oven. Mix well and cool until solidified. Accurately weigh portion of sample equivalent to 6 mg into 150 mL beaker. Add ca 40 mL acidic ethanol and heat on steam bath 10 min with occasional stirring. Cool and quantitatively transfer to 100 mL volumetric flask and dilute to volume with acidic ethanol. Dilute 25.0 mL filtrate to 100.0 mL with acidic ethanol.

Gelatin-encapsulated.—Individual suppository.—Slit capsule along seam with scalpel and extrude contents as completely as possible into 250 mL beaker. Place capsule in beaker and add ca 100 mL acidic ethanol. Heat on steam bath 10 min with occasional stirring and probing of gelatin capsule with glass rod. Quantitatively transfer to 200 mL volumetric flask with acidic ethanol. Repeat extraction with ca 50 mL acidic ethanol. Examine capsule for completeness of content extraction. Discard sample if appreciable residue remains in capsule. Cool and dilute to volume with acidic ethanol. Filter solution, discarding first 10 mL filtrate. Dilute 25.0 mL filtrate to 100.0 mL with acidic ethanol.

Suppository composite.—Treat 5 suppositories as described for individual suppository. Extract with additional 35 mL acidic ethanol. Dilute 25.0 mL filtrate to 100.0 mL with acidic ethanol.

Determination

Scan sample solution and equivalent standard solution from 500 to 320 nm against acidic ethanol blank. Determine maximum absorbance at ca 402 nm and minimum absorbance at ca 412 and 389 nm. Calculate net absorbance for sample and standard from formula:

$$A = A_{402} - \tfrac{1}{2}(A_{412} + A_{389}) \text{ (see Figure 1)}$$

$$\% \text{ Aminacrine hydrochloride in creams and jellies} = (A/A') \times (C/W) \times 10$$

$$\text{mg Aminacrine hydrochloride/molded suppository} = (A/A') \times C \times (S/W) \times 400$$

$$\text{mg Aminacrine hydrochloride/individual gelatin suppository} = (A/A') \times C \times 800$$

$$\text{mg Aminacrine hydrochloride/gelatin suppository composite} = (A/A') \times (C/T) \times 4000$$

where A and A' = net absorbance of sample and standard solutions, respectively; C = mg aminacrine hydrochloride/mL; W = sample weight; S = average suppository weight; and T = number of suppositories in composite.

TLC Identification Test for Aminacrine Hydrochloride

Line suitable chromatographic tank with filter paper and add developing solvent. Dilute sample and standard solutions from assay 1:10 with ethanol. Spot 3 μL diluted sample and

standard solutions on TLC plate. Develop 10 cm above starting line and air dry. Locate and mark spots under longwave UV light. R_f of sample spot corresponds to that of standard spot.

Results and Discussion

Aminacrine hydrochloride is marketed in cream, jelly, and suppository formulations (8). A representative cross section of the available products was collected for this study. Typical constituents of aminacrine hydrochloride formulations are:

Creams—sulfanilamide or sulfisoxazole, allantoin, dienestrol, aminacrine hydrochloride-4-hexylresorcinol, *p*-chloro-*m*-xylenol, and methyl and propyl parabens in hydrophilic bases.

Jellies—oxyquinoline benzoate, butyl-*p*-hydroxybenzoate, and methyl and propyl parabens in a jelly base.

Suppositories—sulfanilamide or sulfisoxazole, allantoin, dienestrol, polyoxyethylene nonyl phenol, sodium dioctyl sulfosuccinate, and methyl and propyl parabens in polyethylene glycol base, either plain or gelatin-encapsulated.

The visible spectra of sulfanilamide, sulfisoxazole, allantoin, dienestrol, and oxyquinoline benzoate were recorded between 500 and 300 nm. In this range, only oxyquinoline benzoate caused a minimal interference in the aminacrine hydrochloride absorption spectrum. The use of the previously described net absorbance at the 402 nm aminacrine hydrochloride maximum in the calculation equations compensates for any interference from the oxyquinoline benzoate spectrum. This technique also eliminates any interference from excipients. Therefore, there is no need for an extraction step prior to recording the absorption spectrum of aminacrine hydrochloride. Linearity for aminacrine hydrochloride was established between 5.2 and 20.8 μg/mL for both the total absorbance and the net absorbance at 402 nm.

Portions of 14 commercial products were analyzed using the proposed method. Results ranged from 92.5 to 109.5% of the labeled amount of aminacrine hydrochloride (Table 1). Six portions of the 5 products listed in Table 2 were analyzed by the proposed method to test its reproducibility. The coefficients of variation (CV) ranged from 0.42 to 1.22%. For the gelatin-encapsulated suppository sample, the close agreement between the CV values for the composite and 3 individual analyses as well as the higher results for the individual analyses indicate that an analysis of 5 individual suppositories is preferable to a composite analysis to obtain accurate results for this dosage form. The CV of 0.39% for the suppository composite shows that the baseline technique for the calculation produces precise results because the only variables introduced

Table 1. Analysis of commercial products for aminacrine hydrochloride

Sample No.	Dosage form	Dosage level	% Label claim	% Label claim[a]
1	cream[b]	0.2%	100.5	—
2	cream[b]	0.2%	101.0	—
3	cream[b]	0.2%	92.5	—
4	cream[b]	0.2%	107.0	106.5
5	cream[c]	0.2%	97.5	—
6	cream[d]	2 mg/mL	105.5	—
7	jelly[e]	0.2%	95.5	98.0
8	cream[f]	0.2%	109.5	—
9	suppository[g]	6 mg	100.5[h]	100.5[h]
10	suppository[b,i]	14 mg	100.0	—
11	suppository[b,i]	14 mg	98.6[h]	97.9[h]
12	suppository[b,i]	14 mg	100.0	—
13	suppository[c,i]	12 mg	97.5	—
14	suppository[f,i]	14 mg	98.6	—
Standard recovery		1.56 mg	—	98.3

[a] Sample cleanup with basic Celite 545 column and methylene chloride elution.
[b] Aminacrine hydrochloride, sulfanilamide, allantoin.
[c] Aminacrine hydrochloride, sulfisoxazole, allantoin.
[d] Aminacrine hydrochloride-hexylresorcinol compound.
[e] Aminacrine hydrochloride, oxyquinoline benzoate.
[f] Aminacrine hydrochloride, sulfanilamide, allantoin, dienestrol.
[g] Molded polyethylene glycol base suppository.
[h] Same single suppository stock solution.
[i] Gelatin-encapsulated polyethylene glycol base, individual suppository analysis.

Table 2. Reproducibility study

Sample No.	Dosage form	Dosage level	% Label claim[a] Range	Av.	CV,%
5	cream	0.2%	94.0–97.5	96.0	1.22
7	jelly	0.2%	95.5–98.0	97.0	0.90
8	cream	0.2%	107.0–110.0	108.5	0.99
9	suppository[b]	6 mg	100.7–102.7	101.8	0.88
14	suppository[c]	14 mg	96.9–97.6	97.4	0.39
14	suppository[d]	14 mg	98.6–99.3	98.6[e]	0.42

[a] Six analyses.

[b] Separate analyses of composite of 5 molded polyethylene glycol base suppositories.

[c] Separate aliquots of stock solution from composite of 5 gelatin-encapsulated polyethylene glycol base suppositories.

[d] Individual analyses of gelatin-encapsulated polyethylene glycol base suppository.

[e] Average of 3 analyses.

after compositing are the aliquot dilution and calculation steps. Aminacrine hydrochloride (1.56 mg) was added to a hydrophilic ointment base (Parke-Davis NDC 0071-3122-23) along with either sulfanilamide (132.7 mg) or sulfanilamide and oxyquinoline benzoate (140 and 1.58 mg). Aminacrine hydrochloride recoveries were 98.2 and 97.9%, respectively.

In a limited study, the proposed direct dilution method was compared with a possible Celite column cleanup to investigate the necessity of such a procedure. The column procedure incorporated a 1N NaOH Celite 545 column with a methylene chloride elution. The eluate was evaporated to dryness and the residue was dissolved in acidic ethanol. Results were calculated by the net absorbance technique described for the proposed method. Results are listed in Table 1. With the exception of the jelly sample, the results for the 2 methods were equivalent. Standard recovery was 98.3%. This study showed that prior cleanup did not improve the analytical results but did increase the time to perform the analysis.

Aminacrine hydrochloride was confirmed by TLC. The sample spots for aminacrine hydrochloride had the same R_f as the standard spot and were resolved from the other drugs that are incorporated into commercial formulations. If fluorescent indicator plates are used, the other drugs, except for allantoin, can also be visualized. Allantoin is only slightly soluble in alcohol and appeared to be the white residue remaining in the stock sample solutions. Its concentration in the final sample dilution was too low to be detected. Aminacrine hydrochloride was located as a blue fluorescent spot under longwave UV light. The R_f was about 0.7 under the experimental conditions.

A trace contaminant, 9(10*H*)-acridone, was found in 2 samples of aminacrine hydrochloride and 1 sample of aminacrine base. Acridone was also detected in the commercial formulations. Assuming equivalent quantum efficiency for 9-acridone and 9-aminacrine hydrochloride, acridone was present in the standard at about 0.4% after isolation by TLC and quantitation by fluorescence spectroscopy. Commercial aminacrine hydrochloride is prepared from 9-chloroacridine (1). However, 9-chloroacridine is readily converted to acridone by heating an aqueous solution of the material (9). Similarly, alcoholic solutions of 9-chloroacridine will convert to acridone on standing overnight. Given the instability of the material, it is not surprising that a bottle of 9-chloroacridine purchased in 1970 was approximately 50% acridone, while a bottle purchased in 1982 had a substantially lower level of acridone. From this small sample, it is apparent that 9-acridone is a common contaminant of 9-chloroacridine. Acridone can be resolved from 9-aminoacridine and 9-chloroacridine by TLC. On Eastman silica gel Chromagram sheets (No. 6061), with a developing solvent of benzene–methanol (95 + 5) (5), the respective R_f values are 0.2, 0, and 0.6. On silica gel-coated glass plates with a developing solvent of methylene chloride–diethylamine (2 + 1), the respective R_f values are 0.4, 0.6, and 0.8. The infrared spectrum of 9-acridone synthesized from 9-chloroacridine matched that of 9(10*H*)-acridone purchased from Eastman Kodak Co. Both compounds have the same R_f as the trace spot in aminacrine hydrochloride when chromatographed with the systems listed above. Acridone is a consistent contaminant in aminacrine and can be directly traced to the 9-chloroacridine synthesis precursor.

The proposed method is rapid and accurate and can be used for the determination of aminacrine hydrochloride in combination with other drugs in commercial products.

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Evaluation of Fluorometric Determination-Thin Layer Chromatographic Identification of Aminacrine Hydrochloride in Drug Preparations

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Utilizing the fluorescent property of aminacrine hydrochloride and a filter fluorometer, a fluorometric method for aminacrine hydrochloride in drug combinations was developed, collaboratively studied, and adopted as official first action in the 11th edition of *Official Methods of Analysis*. Identity was confirmed by thin layer chromatography (TLC). Additional analytical work was undertaken with a grating fluorometer to support a change in the method status to official final action. The grating instrument recorded the aminacrine hydrochloride spectrum as opposed to the total fluorescence emission measured by the filter instrument. The spectrum of aminacrine hydrochloride showed that the molecule was exhibiting self-absorption of the emitted radiation even at concentrations of 10^{-6}M and that the ratio of the 2 peaks in the emission spectrum varied with concentration. Additional analyses of an authentic cream preparation that also contained sulfanilamide gave an average recovery of 86.0% for aminacrine hydrochloride in 10 replicate portions. Because of these observations, the current Associate Referee's recommendation to delete the fluorometric procedure from the 13th edition of *Official Methods of Analysis* was adopted. A recommended deletion of the TLC identification test was also adopted.

Aminacrine hydrochloride (9-aminoacridine) is used extensively as an active component in drug preparations used to treat vaginal infections. At the time of the original method development, aminacrine as either its hydrochloride, propionate, or undecylenate salt was formulated into tablets, powders, suppositories, creams, jellies, tampons, and ointments (1, 2). At present, however, aminacrine is marketed only as the hydrochloride salt in either creams, jellies, or suppositories. The final drug product always contains other active ingredients, the most common of which are sulfanilamide, sulfisoxazole, allantoin, oxyquinoline benzoate, and dienestrol (3). Aminacrine is used because of its antiseptic properties. It possesses a broad bactericidal spectrum and is effective against anaerobes, species of the proteus group, and other Gram-positive and Gram-negative bacilli. The mechanism of action is attributed to its ability to form nonionizing or feebly ionizing complexes with the acidic groups of nucleoproteins in the bacteria cytoskeleton (4–6).

Fluorescence spectroscopy seemed an ideal technique to use to quantitate aminacrine hydrochloride in the presence of other compounds because it has a quantum efficiency of 0.98 compared with that of 0.55 for quinine sulfate, a substance that has been widely used as an instrumental reference standard (7). Indeed, the method adopted for the 11th edition of *Official Methods of Analysis* (8) relied on the fluorescent properties of aminacrine for quantitation. In this procedure the sample is dissolved in alcohol and an aliquot equivalent to 1 μg aminacrine hydrochloride is treated with 5% NaOH. A chloroform extract is evaporated and the residue is redissolved in acidic ethanol before the fluorescence measurement. A thin layer chromatographic (TLC) procedure (benzene–methanol (95 + 5), silica gel), described by Stahl (9) for the resolution of acridine, is used for confirmation. The spot(s) is visualized under ultraviolet (UV) light. The collaborative study of the method included an authentic powder and cream prepared by the former Associate Referee (2). The General Referee for Nonalkaloid Organic Nitrogenous Bases found a mathematical error in the statistical evaluation of the collaborative study and requested additional work by the author (current Associate Referee) to support a change in the method status to official final action in the compendium. Both the quantitative and qualitative procedures were evaluated as a result of this request.

Quantitative Evaluation

The excitation and emission spectra of aminacrine hydrochloride in acidic alcohol were recorded with a grating spectrophotofluorometer. Excitation maxima were at 268, 410, and 430 ± 2 nm with a shoulder at ca 380 nm. The emission spectrum had 2 maxima at 435 and 458 ± 2 nm (see Figure 1). The 435 nm maximum was arbitrarily chosen for analytical calculations. According to sec. **38.110** (10), a pri-

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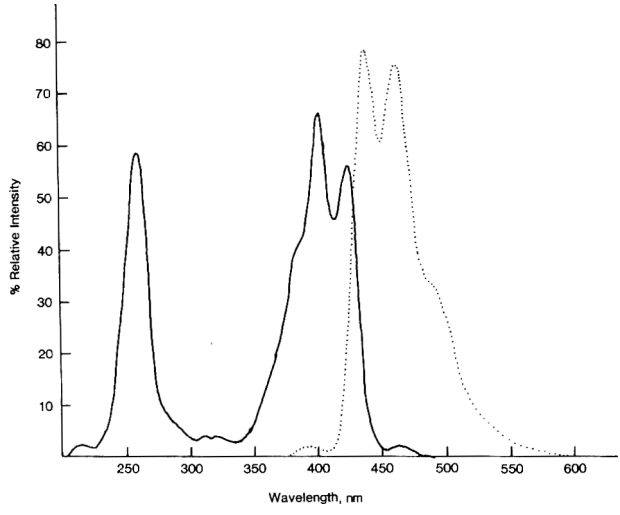

Figure 1. Uncorrected fluorescence spectra of aminacrine hydrochloride in acidic ethanol. Solid line, excitation spectrum (emission wavelength 460 nm); dotted line, emission spectrum (excitation wavelength 390 nm).

mary filter passing light at 365 nm (Corning No. 7-39) and a secondary filter with a cutoff wavelength at 415 nm (Wratten 2A) were to be used with a filter fluorometer for excitation and emission, respectively. The 415 nm cutoff filter allowed the photomultiplier tube in the filter fluorometer to see all of the emitted energy from both the 435 and 458 nm peaks. The grating instrument records the individual emitted energy for each maximum. Inspection of the emission and excitation spectra showed that there was an area coincident to both spectra between ca 415 and 440 nm. The importance of this observation, which cannot be made with a filter instrument, was demonstrated during the evaluation of the analytical method.

Four commercial products, a cream, a jelly, and a molded and a gelatin-encapsulated suppository, were selected for analysis for aminacrine hydrochloride. These products also contained the following active drug ingredients: sulfanilamide, allantoin, oxyquinoline benzoate, tyrothricin, and phenylmercuric acetate. (The product containing phenylmercuric acetate and tyrothricin has since been discontinued.) Each product was analyzed in triplicate with final dilution to 1.0 μg/mL. For the gelatin-encapsulated suppository, 1 suppository was dissolved in alcohol and 3 aliquots were taken for analysis. The results of the analyses are shown in Table 1. The average recovery based on the label declaration was 91.5–101.3%, while the coefficients of variation (CV) ranged from 0.50 to 6.08%. The 2 samples with the highest CVs, 1.12 and 6.08%, also contained sulfanilamide. The 1.12% CV resulted from the analysis of 3 aliquots from the solution of the 1 gelatin-encapsulated suppository. It is unlikely that the 6.08% CV for the cream was due to nonhomogeneity of the sample because it was thoroughly mixed before analysis. The 0.50% CV for the jelly sample supports the hypothesis that a mechanism other than sampling error is responsible for the high CV. Six replicate analyses by a visible spectrophotometric method gave CV values of 0.70% for a different

Table 1. Analysis [a] of commercial products for aminacrine hydrochloride by secs. 38.111–38.113 (10) using a grating spectrophotofluorometer

	Sample			
	1 [b]	2 [c]	3 [d]	4 [e]
	99.5	97.5	101.7	96.4
	99.0	86.5	100.7	97.1
	100.0	90.5	102.0	98.6
Av.	99.5	91.5	101.3	97.1
CV, %	0.50	6.08	0.68	1.12

[a] Results expressed as percent of label declaration.

[b] 2 mg aminacrine hydrochloride/g and 7.5 mg oxyquinoline benzoate/g in a jelly base.

[c] 0.2% aminacrine hydrochloride, 15.0% sulfanilamide, and 2.0% allantoin in a cream base.

[d] 3.0 g aminacrine hydrochloride, 2.5 mg tyrothricin, 3.0 mg phenylmercuric acetate, and 20.0 mg urea in each molded, water-soluble, wax-based suppository.

[e] 14 mg aminacrine hydrochloride, 1.05 g sulfanilamide, and 140 mg allantoin in each gelatin-encapsulated polyethylene glycol-based suppository.

Table 2. Recovery (%) of aminacrine hydrochloride in a cream base [a]

	0.08 μg/mL Standard		1.0 μg/mL Standard		
Sample	RF[b]	Found	RF[b]	Found[c]	Found[d]
Std	65.3		58.3		
1[e]	62.2,	95.6,	63.4,	136.5,	97.1,
	68.9	98.6	69.9	140.3	100.0
2[f]	68.1,	98.7,	69.1,	140.3,	100.2,
	61.9	95.4	63.1	135.8	97.1

[a] Fluorescence of duplicate sample solutions was recorded with each of the 2 standard dilutions; the grating spectrophotofluorometer settings were identical: excitation 390 nm, emission 435 nm.

[b] Relative fluorescence as percent full-scale recorder deflection.

[c] Calculated with the 1.0 μg/mL standard.

[d] Calculated with the 0.08 μg/mL standard.

[e] Cream containing 2.08 mg aminacrine hydrochloride/g.

[f] Cream containing 14.03 mg aminacrine hydrochloride/g.

lot of jelly and 1.22 and 0.99% for 2 creams. The 2 creams also contained sulfisoxazole and allantoin, and sulfanilamide, allantoin, and dienestrol, respectively.

Three authentic samples were prepared. Aminacrine hydrochloride alone was incorporated into a commercial cream at 2 levels, 2.08 and 14.03 mg/g. For the third sample, 500 mg sulfanilamide/g and 2.0 mg aminacrine hydrochloride/g were combined with a commercial ointment base. Recoveries for the 14.03 mg/g sample ranged from 90.8 to 98.7% for 4 replicate samples; recoveries for the 2.08 mg/g sample were 95.6 and 98.6% for duplicate samples. Duplicate portions of the 2.08 and 14.03 mg/g samples were analyzed at the same time with a final sample concentration of ca 0.08 μg/mL. The fluorescence of the sample solutions was recorded first with a 0.08 μg/mL standard and then a 1.0 μg/mL standard, using the same instrumental parameters. The observation that the relative fluorescence intensity of the 1.0 μg/mL standard was 7% less than that of the less concentrated standard suggested that concentration quenching might be occurring. The relative fluorescence of sample and standard solutions as well as calculations based on both standard readings are shown in Table 2. Ten replicate portions of the third authentic sample, which also contained sulfanilamide, were analyzed at dilutions of 0.08 and 1.0 μg/mL. The range of recoveries at the 0.08 μg/mL dilution was 78.0–90.5% with an average of 86.0% and a CV of 4.71%; for the 1.0 μg/mL dilution the recoveries were 76.0–92.0% with an average of 86.0% and a CV of 5.44%. The larger CV and wider range for the 1.0 μg/mL dilution indicate potential problems directly associated with concentration. At concentrations of 0.08 and 1.0 μg/mL, aminacrine hydrochloride is 3.5×10^{-7} and 4.3×10^{-6}M, respectively.

Table 3. Change in ratios of 435/458 nm emission maxima with change in concentration of aminacrine hydrochloride in acidic ethanol

μg/mL	10^{-6}M	Excitation 390 nm
0.198	0.86	1.051
0.396	1.7	1.038
0.593	2.6	1.027
0.791	3.4	1.027
0.989	4.3	1.026
1.484	6.4	1.007
1.978	8.6	0.994
2.472	10.8	0.983
2.967	12.8	0.975

Emission linearity was checked between 0.198 μg/mL (8.6×10^{-7}M) and 2.967 μg/mL (1.3×10^{-5}M). The line equation and correlation coefficient (r) for the 435 nm maximum were $y = 24.2x + 2.9$, $r = 0.99871$; for the 458 nm maximum, $y = 25.1x + 1.8$, $r = 0.99947$. The ratio of the emission peaks, 435/458 nm, changed from 1.051 for 0.198 μg/mL to 0.975 for the 2.967 μg/mL concentrations (excitation wavelength 390 nm). The change in the relative intensity of the 2 peaks is directly related to concentration (Table 3).

Qualitative Evaluation

Confirmation was attempted by TLC. Standard solutions were prepared according to sec. **38.115** (10), spotted on Eastman No. 6060 silica gel with fluorescent indicator TLC sheets, and developed with benzene–methanol (95 + 5). This TLC system was reported by Stahl (9) for acridine and other organic bases derived from tar. Aminacrine hydrochloride is a brilliant yellow compound, and the 3 μg spotted for this test leaves a visible yellow spot at the origin. After development of the TLC plate, the yellow spot remains at the origin. As described in sec. **38.115** (10), the second minor spot to be used for the identification of aminacrine is located under UV light about midway up the plate. Both spots exhibit bright blue fluorescence. Chromatography was repeated with glass plates precoated with silica gel. Subsequently, the minor spot was identified as 9(10*H*)-acridone, while the intense baseline spot was identified as aminacrine. The minor spot (9(10*H*)-acridone) appeared to be

a common and consistent contaminant in aminacrine hydrochloride at ca 0.3%. Acridone is easily derived from 9-chloroacridine, the immediate precursor in the synthesis of aminacrine hydrochloride. Although this is an excellent identification test for 9(10*H*)-acridone, it misidentifies aminacrine hydrochloride. On the recommendation of the Associate Referee, this test was deleted from the 13th edition of *Official Methods of Analysis* (11).

Discussion

Proflavin (3,6-diaminoacridine hydrochloride), a member of the acridine dye family, exhibits self-quenching even in 10^{-6}M solutions at pH 4 (12). This behavior is independent of solution viscosity and temperature. More recent data indicate that the self-quenching observed for the acridine family is due to self-absorption of the fluorescent radiation (13). This theory fits the observed shift in the emission peak ratios noted in Table 3. At 0.86×10^{-6}M, the ratio of 435/458 nm is 1.051. This ratio changes to 0.975 as the concentration increases to 12.8×10^{-6}M. With a 15-fold increase in concentration, the peak ratios decrease by 7%. As concentration increases, the 435 nm emission peak becomes the smaller of the pair. Quenching due to self-absorption would account for this phenomenon. Although the correlation coefficient from the linear regression of the linearity study is acceptable for either peak maximum, that for the 458 nm peak is slightly better (0.99945 compared with 0.99871) and the line passes through the abscissa at 1.8 rather than 2.9. However, if the actual plotted line is examined, the line diverges from the theoretical as concentration becomes >1 μg/mL. This deviation is greater for the 435 nm maximum. Other solvents were evaluated to determine if this self-absorption could be eliminated by means other than dilution to $<10^{-6}$M. The results indicated that, similar to the findings of Millich and Oster (12), the phenomenon appears to be independent of solvent effects.

The amount of aminacrine hydrochloride recovered from either a cream or ointment base has a random bias. The cream, which was fortified with only aminacrine hydrochloride at 2.08 or 14.03 mg/g, gave average recoveries of 97.1 (2 analyses) and 95.4% (4 analyses), respectively. For the 2.08 mg sample, recoveries were 95.6% and 98.6%. The high recovery for the 14.03 mg sample was 98.7% and the low 90.8%. The recovery of aminacrine hydrochloride prepared with sulfanilamide in an ointment base dropped to 86.0% with a high of 92.0% and a low of 76.0% for the 10 analyses. As the number of samples increased so did the range of the results. The former Associate Referee's collaborative study had 8 participants with a total of 16 cream analyses. This authentic sample also contained sulfanilamide and allantoin. If the paper presenting the collaborative study results does not contain a misprint for the amount of aminacrine hydrochloride incorporated into the authentic sample, the average recovery was not the 102% reported but 79.6%. None of the results can be discarded as outliers based on the criteria of Dixon (14). The range for this study was 40.9%, calculated from the data presented. The collaborative study reports a 100% recovery of the 0.108 mg/g added to the authentic powder that did not contain sulfanilamide. The range for the powder was still 24.6% for 16 analyses. The CV for the powder sample was 6.55%, compared with 10.68% for the cream. These analytical results indicate that a random bias is associated with the chloroform extraction of the basic sample solution. While a slight shift in the excitation grating can also cause a random error due to changes in the intensity of the excitation energy, it is more likely that the observed random results are due to other factors because the collaborators used filter instruments, which measure the total fluorescence energy rather than that at a single wavelength. There was a trend toward lower recoveries as the time in contact with 5% NaOH increased; the lowest result was also the last sample in the series to be extracted. Some sulfanilamide is not removed during the cleanup procedure. The commercial products that contained sulfanilamide and allantoin also had higher CV values.

A different source of error is observed if an excitation maximum at 270 nm rather than 390 nm is used. The fluorescence of aminacrine hydrochloride decreases as the amount of sulfanilamide in the solution increases and an additional emission peak at about 345 nm is also observed. The recovery for one sample combining aminacrine hydrochloride, sulfanilamide, and ointment base was 90.0% with a 270 nm excitation wavelength, compared to 95.0% with a change to 390 nm. The 95.0% result was verified by visible spectrophotometry. Figure 2 illustrates the quenching of the aminacrine fluorescence spectrum when up to 7.63 mg sulfanilamide was added to 0.1 mg aminacrine hydrochloride/100 mL acidic alcohol. The ratio of sulfanilamide to aminacrine hydrochloride in commercial formulations is 75:1. With an excitation wavelength of 270 nm the relative fluo-

rescence decreased from 63.7% for aminacrine hydrochloride alone to 9.5% with 7.63 mg sulfanilamide added to the solution. When the excitation wavelength was changed to 390 nm, the same solutions produced a ± 2% random variation in the relative fluorescence around that recorded for aminacrine hydrochloride alone. The wavelengths for the aminacrine hydrochloride emission maxima remain unchanged with either excitation wavelength.

Acridine dyes have been used extensively in the analysis of nucleic acids by measuring the difference between the fluorescence of the unbound dye and the quenched fluorescence of the nucleic acid-dye complex. Although the fluorescence of acridine orange (2,8-dimethylaminoacridine) at 10^{-3}M is only 1% of that at 10^{-5}M, the quenching is never more than 20% of that observed without the nucleic acid (13). Georghiou (15) reports that the fluorescent quenching and decay times for the complexes between nucleotides and 9-aminoacridine (aminacrine) have been studied. Georghiou (15) also discusses the role of hydrogen bonding in the formation of complexes between nucleic acids and acridine dyes. He further notes that the fluorescence of proflavin and acridine orange was strongly quenched by hydrogen bonding with phenol and concludes that this is due to charge transfer between the aromatic rings through the hydrogen bonds betweeen substituent groups on the rings. Sulfanilamide could form hydrogen bonds with aminacrine hydrochloride. Experimental results indicate that there are at least 3 phenomena which individually or in combination affect the accuracy of the fluorometric assay: (*1*) self-absorption of the fluorescent radiation, (*2*) chloroform extraction of the basic sample solution, and (*3*) formation of a sulfanilamide-aminacrine hydrochloride complex. Allantoin does not appear to influence analytical results when the results of analysis of authentic samples with either aminacrine alone or aminacrine with sulfanilamide are compared with those for commercial products. This may be due in part to the low solubility of allantoin in alcohol or the fact that it does not appear to be a fluorophore as is sulfanilamide.

Of all the products containing aminacrine hydrochloride that are currently available, only one, a jelly, contains neither sulfanilamide nor sulfisoxazole. Even if dilution of aminacrine hydrochloride were able to solve the problem of linearity changes due to the self-absorption of emitted radiation, the method would not produce accurate results. Both sulfanilamide and

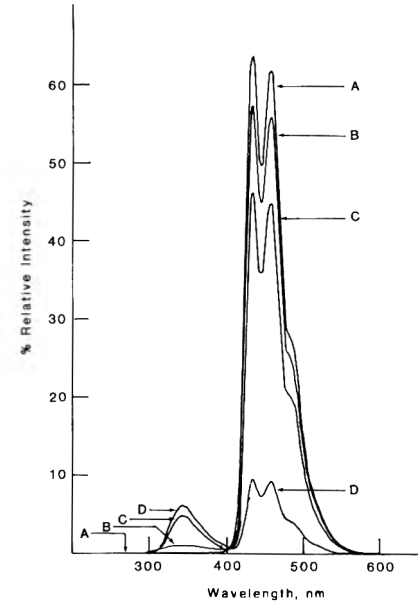

Figure 2. Uncorrected emission spectra of acidic ethanol (1 + 99) solutions of aminacrine hydrochloride (I) and sulfanilamide (II) with excitation at 270 nm. A, 1.0 μg I/mL; B, 100 μg I, 7.63 mg II, and 100 mg ointment base extracted per sec. 38.112 (10). Final volume 100 mL; C, 1.0 μg I/mL and 12.7 μg II/mL; D, 1.0 μg I/mL and 76.3 μg II/mL.

sulfisoxazole could form hydrogen-bonded complexes with aminacrine. The resulting complex quenches the fluorescence of aminacrine and produces a constant negative bias in the analytical results, thereby precluding the use of the 270 nm excitation wavelength. Although the fluorescent properties of aminacrine seemed to provide an excellent technique for its assay, the phenomenon of self-absorption of the emitted fluorescent energy coupled with the tendency to form fluorescence quenching complexes and the apparent random extraction bias make it impossible to use this property for quantitative analyses. The fluorometric method for aminacrine hydrochloride was deleted from the 13th edition of *Official Methods of Analysis* (11) for these reasons. The TLC identification test was also deleted because 9(10*H*)-acridone was misidentified as aminacrine and aminacrine had an R_f of 0 in this system.

Subsequent to this study, a visible spectrophotometric method was developed to quantitate aminacrine hydrochloride in combination with the drugs and formulations listed here. A new TLC identification test is also presented in the paper that describes this spectrophotometric method (16).

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Liquid Chromatographic Analysis of Samples Containing Cocaine, Local Anesthetics, and Other Amines

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A liquid chromatographic procedure is described for the separation of cocaine, local anesthetics, and other amines often encountered in cocaine look-alike preparations. Amines such as ephedrine, pseudoephedrine, and phenylpropanolamine are identified following derivatization with phenylisothiocyanate. The isocratic, reverse phase method uses dual wavelength detection at 254 and 280 nm.

The stimulant and local anesthetic properties of cocaine have made this drug one of the most frequently abused substances encountered in forensic laboratories. The illegal use of cocaine has prompted much research into the analysis of cocaine and related compounds by gas chromatography (1), high pressure liquid chromatography (HPLC) (2), and infrared, nuclear magnetic resonance, and mass spectrometry (3).

Recently, numerous samples suspected of containing cocaine have been submitted for analysis to the Alabama Department of Forensic Sciences. Many of the samples were found to contain cocaine in combination with lidocaine, benzocaine, procaine, and amphetamine. However, a number of other suspected cocaine samples were found to contain only local anesthetics in combination with various amines. Among the drugs that have been encountered in combination are (a) lidocaine, tetracaine, ephedrine, and phenylpropanolamine; (b) procaine, lidocaine, and phenylpropanolamine; (c) lidocaine, ephedrine, and caffeine; and (d) tetracaine, lidocaine, pseudoephedrine, and caffeine. Intelligence information indicates that the local anesthetic–amine combinations, commonly called cocaine look-alikes, are readily available under such names as Pseudo-Caine, Super Caine, and Sno-Caine.

Liquid chromatography is an especially useful technique for separating polar, nonvolatile, or thermally unstable compounds such as cocaine, benzoylecgonine, and related compounds. Previously, a procedure was described in which cocaine, benzoylecgonine, and *cis*- and *trans*-cinnamoylcocaine were separated by liquid chromatography, with identification based on retention data, absorbance ratios at 254 and 280 nm, and mass spectral properties (4). In this paper, the method has been expanded to demonstrate usefulness of the procedure for separating commonly occurring local anesthetics in combination with cocaine, as well as in analyzing cocaine look-alike preparations. In addition, the usefulness of a previously described method for detecting primary and secondary amines found in cocaine look-alikes by HPLC, using phenylisothiocyanate as derivatizing agent to enhance detectability and chromatography, is also demonstrated (5).

Experimental

Reagents and Chemicals

trans-Cinnamoylcocaine and benzoylecgonine were obtained from the Drug Enforcement Administration, Special Testing and Research Laboratory, McLean, VA. Other drugs, with the exception of *cis*-cinnamoylcocaine which is not commercially available, were obtained from their representative manufacturers and used without further purification. *cis*-Cinnamoylcocaine was present in illicit cocaine samples. HPLC grade methanol and phenylisothiocyanate (PIT) were purchased from Fisher Scientific Co., Fair Lawn, NJ. Illicit cocaine samples and cocaine look-alike preparations were submitted to the Alabama Department of Forensic Sciences by various Alabama law enforcement agencies.

Instrumentation

The liquid chromatograph consisted of a Waters Associates (Milford, MA) Model 6000A pump, Model U6K injector, Model 440 UV detector with dual wavelength accessory operated at 254 and 280 nm, and a Houston Instrument (Austin, TX 78753) OmniScribe dual pen recorder.

Chromatographic Procedures

All separations were carried out on a 30 cm × 3.9 mm id μBondapak C_{18} column (Waters Associates) at ambient temperature. The analytical column was preceded by a 7 cm × 2.1 mm id guard column dry packed with CO:PELL ODS (Whatman Inc., Clifton, NJ). Powdered samples

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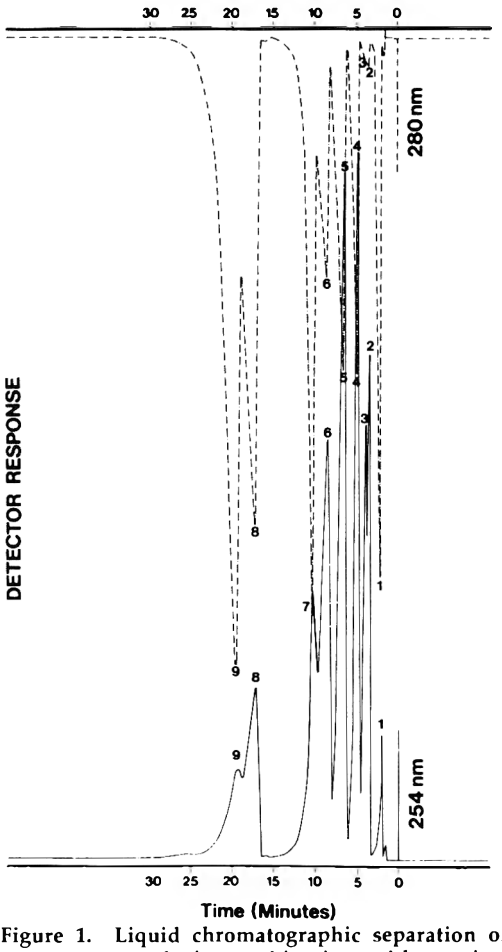

Figure 1. Liquid chromatographic separation of some compounds in combination with cocaine. Peaks: 1, procaine; 2, lidocaine; 3, mepivacaine; 4, benzoylecgonine; 5, cocaine; 6, piperocaine; 7, benzocaine; 8, *trans*-cinnamoylcocaine; 9, tetracaine. Mobile phase, Solvent a.

were dissolved in HPLC grade methanol and initially chromatographed using a mobile phase of pH 3.0 phosphate buffer–HPLC grade methanol (2 + 1) (Solvent a). The pH 3.0 phosphate buffer was prepared by mixing 9.2 g monobasic sodium phosphate (NaH_2PO_4) in 1 L double-distilled water, and adjusting the pH to 3.0 with 2N H_3PO_4. The mobile phase flow rate was 2.0 mL/min. Absorbance ratios (A_{254}/A_{280}) were calculated from the average peak height measurements of a minimum of 3 injections for each drug tested. Potassium permanganate ($KMnO_4$) oxidations were made by adding 5% aqueous $KMnO_4$ solution to the powdered sample in HPLC grade methanol and letting the resulting solution stand 15 min at room temperature before injection.

Table 1. Retention and absorbance data for components of cocaine and cocaine look-alike samples

Drug	Relative retention time	A_{254}/A_{280}
Acetaminophen	0.31	5.84
Procaine	0.32	0.22
Phenylpropanolamine	0.33	very high
Ephedrine	0.34	very high
Pseudoephedrine	0.34	very high
Lidocaine	0.58	20.20
Prilocaine	0.58	13.79
Caffeine	0.58	0.70
Mepivacaine	0.59	21.25
Benzoylecgonine	0.78	2.00
Cocaine	1.00 (12.8 mL)	2.00
Piperocaine	1.41	1.73
Benzocaine	1.59	0.32
cis-Cinnamoylcocaine	1.86	0.76
trans-Cinnamoylcocaine	2.81	0.37
Tetracaine	3.09	0.11
Dibucaine	11.56	2.14

To demonstrate the enhanced detectability and chromatographic separation of primary and secondary amines (ephedrine, pseudoephedrine, and phenylpropanolamine) using phenylisothiocyanate, 0.1000 g cocaine look-alike sample was dissolved in 10 mL 0.5N $NaHCO_3$. A 10 μL injection of this solution was chromatographed at 0.2 AUFS using Solvent a. The remaining portion of the solution was placed in a separatory funnel and extracted twice with 15 mL portions of methylene chloride, and the extracts were combined. PIT (10 μL) was added to the combined methylene chloride extracts which were evaporated to dryness under a stream of air. The residue was dissolved in 10 mL HPLC grade methanol. A 10 μL portion of the solution was chromatographed using a mobile phase of water–methanol–acetic acid (54:45:1) (Solvent b) at a flow rate of 2.0 mL/min and 2.0 AUFS.

Results and Discussion

The HPLC analysis of illicit cocaine samples reveals the presence of several UV-absorbing compounds. The source of the majority of illicit cocaine is believed to be from extraction of the South American coca plant which contains the alkaloids (−)-cocaine, cinnamoylcocaine, the truxillines, and tropacocaine (6). The presence of these alkaloids in illicit cocaine samples can be a significant factor in determining the origin and background of the sample. In a previous report (4), a method was presented for the HPLC separation and identification of cocaine, benzoylecgonine, and *cis*- and *trans*-cinnamoylco-

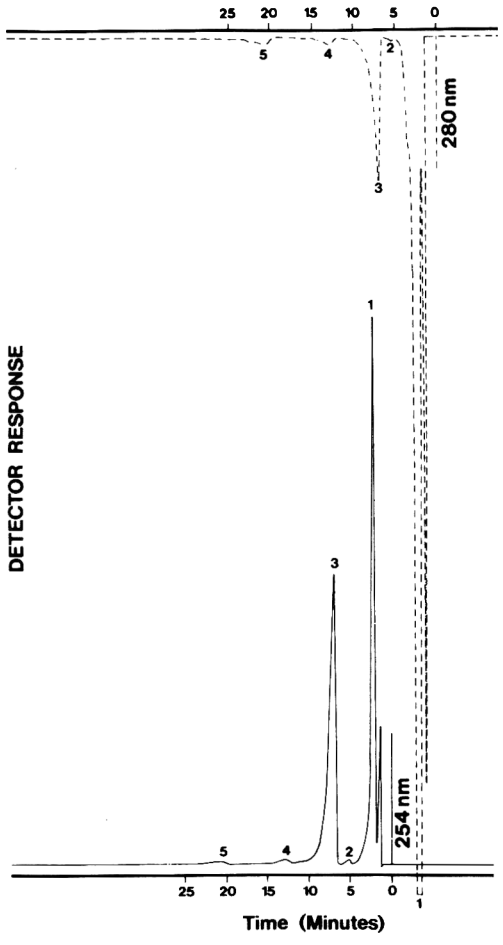

Figure 2. Liquid chromatogram of illicit cocaine sample. Peaks: 1, procaine; 2, benzoylecgonine; 3, cocaine; 4, *cis*-cinnamoylcocaine; 5, *trans*-cinnamoylcocaine. Independent quantitation of this sample showed 20% w/w cocaine. Mobile phase, Solvent a.

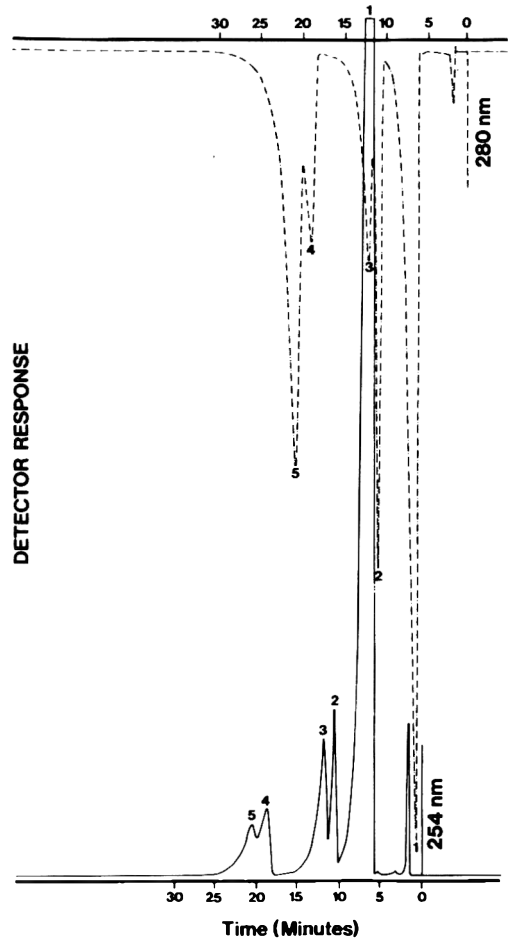

Figure 3. Liquid chromatogram of clandestine sample containing cocaine and local anesthetics. Peaks: 1, cocaine; 2, benzocaine; 3, *cis*-cinnamoylcocaine; 4, *trans*-cinnamoylcocaine; 5, tetracaine. Mobile phase, Solvent a.

caine. The reverse phase (C_{18}) separation of these alkaloids was maximized by using an isocratic solvent system consisting of a 2:1 mixture of pH 3.0 phosphate buffer and methanol. This report examines the application of the procedure to the analysis of various samples containing local anesthetics and amines in combination with cocaine.

The reverse phase HPLC separation of cocaine and related alkaloids from some common local anesthetics is shown in Figure 1. The chromatographic effluent was monitored at 2 wavelengths (254 and 280 nm) using dual flow cells connected in series. Peaks 1-3 in Figure 1 illustrate quite well the advantages of using dual wavelength detection. Procaine (peak 1) shows strong absorbance at 280 nm while lidocaine and mepivacaine (peaks 2 and 3, respectively) give strong absorbances at 254 nm and much less at 280 nm. The chromatographic conditions used for this separation are the same as those reported earlier (4). The relative retention data for the individual components are shown in Table 1. With the exception of dibucaine, all compounds evaluated in this study eluted at a volume of less than 40 mL mobile phase. Benzocaine (pKa 2.8) is the only compound in Figure 1 that would not be totally ionized in the acidic mobile phase. The other basic amines have pKa values (7) in the range 7.5-8.8, and should be completely protonated under these chromatographic conditions.

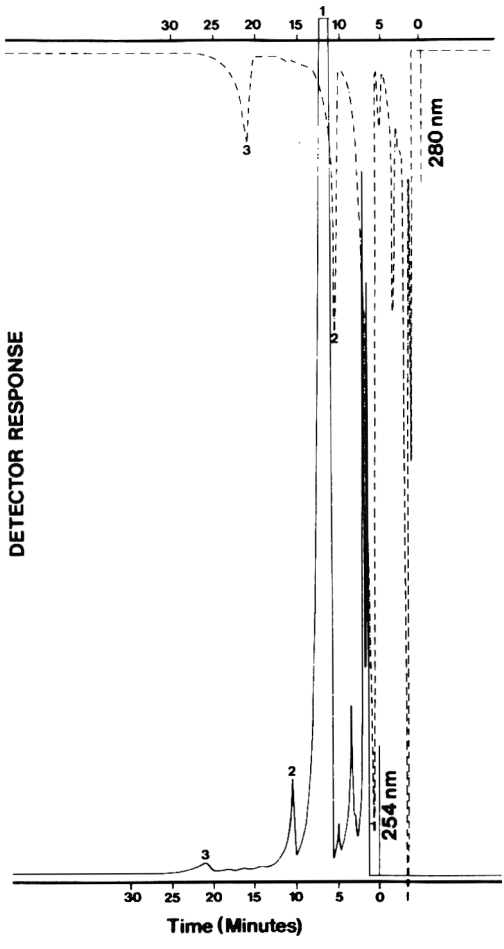

Figure 4. Liquid chromatogram of cocaine sample in Figure 3, after potassium permanganate oxidation. Peaks: 1, cocaine; 2, benzocaine; 3, tetracaine. Mobile phase, Solvent a.

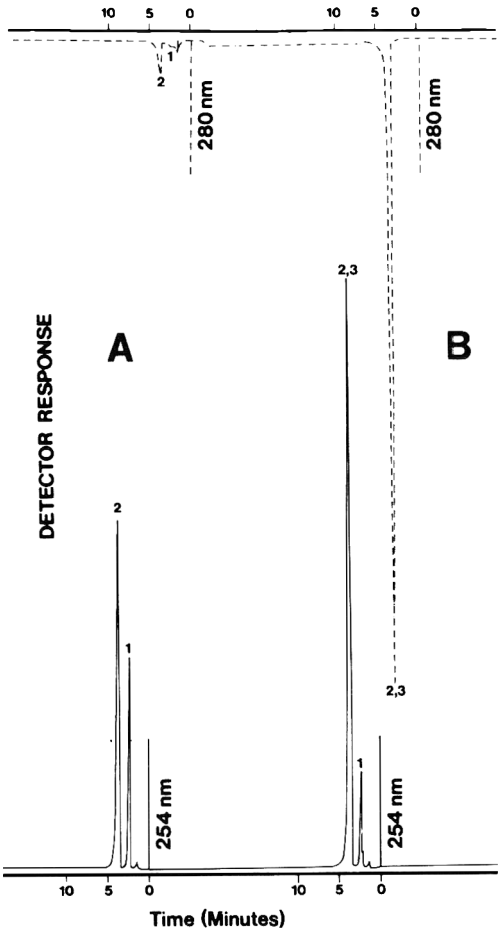

Figure 5. Liquid chromatogram of some cocaine look-alike samples. Peaks: 1, ephedrine; 2, lidocaine; 3, caffeine. Mobile phase, Solvent a.

In addition to retention data, absorbance ratios can provide information concerning the identity of the individual chromatographic peaks. Baker et al. (8) used absorbance ratios to determine the identity of drugs having similar elution characteristics in an HPLC system. The peak height ratios (A_{254}/A_{280}) for all the compounds evaluated in this study are included in Table 1. These ratios should be determined in an individual chromatographic system using reference standards because molar absorptivity and the wavelength of maximum absorbance vary with solvent composition, pH, and other factors (9). To obtain maximum precision, absorbance ratios should only be measured for completely resolved peaks because peak overlap indicates a mixture of continuously changing proportions. The absorbance obtained in such cases is the total absorbance from a 2 or more component mixture. It would seem unlikely for 2 partially resolved compounds to have identical absorbance ratios. Therefore, measurements from partially resolved peaks can yield misleading ratios.

Examples of separations of samples containing combinations of cocaine and local anesthetics are given in Figures 2 and 3. The sample in Figure 2 was found to be a mixture of procaine, cocaine, and the other minor alkaloids of the coca plant. Independent quantitation showed that this sample contained 20% cocaine. The sample analyzed in Figure 3 contains the coca alkaloids, benzocaine and tetracaine. *cis*-Cinnamoylcocaine (peak 3 in Figure 3) was not included in the example separation in Figure 1; however, Figure 3 shows this alkaloid eluting between benzo-

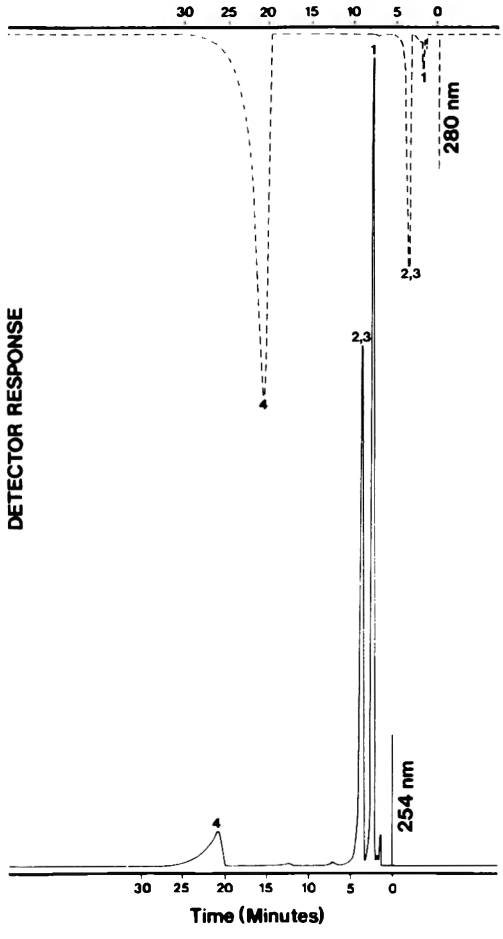

Figure 6. Liquid chromatogram of commonly encountered cocaine look-alike sample. Peaks: 1, pseudoephedrine; 2, lidocaine; 3, caffeine; 4, tetracaine. Mobile phase, Solvent a.

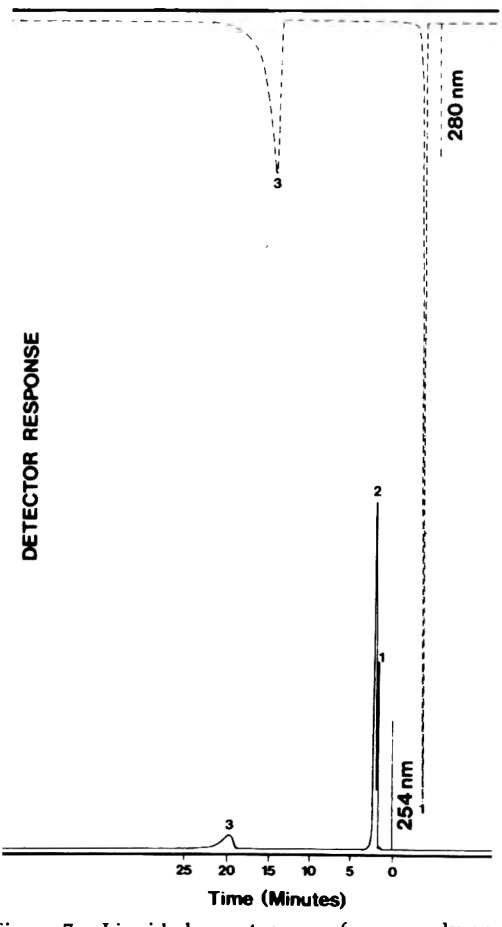

Figure 7. Liquid chromatogram of commonly encountered cocaine look-alike sample. Peaks: 1, procaine; 2, phenylpropanolamine; 3, tetracaine. Mobile phase, Solvent a.

caine and *trans*-cinnamoylcocaine (peaks 2 and 4 in Figure 3; peaks 7 and 8 in Figure 1).

The chromatogram in Figure 4 presents the results of mild potassium permanganate oxidation of the sample in Figure 3. The peaks for *cis*- and *trans*-cinnamoylcocaine from Figure 3 are no longer present in the oxidized sample and the lower intensity of the remaining peaks is the result of dilution. Olefinic double bonds such as those found in the cinnamoyl group are particularly susceptible to oxidation by potassium permanganate. The unnumbered early eluting peaks in Figure 4 are the olefin oxidation products and the UV-absorbing permanganate ion. Potassium permanganate in the presence of background electrolyte in the mobile phase has been shown (10) to elute at approximately the column void volume.

Figures 5–7 are examples of separations of commonly encountered cocaine look-alike products. These products are usually mixtures of local anesthetics, caffeine, and amines such as ephedrine, pseudoephedrine, or phenylpropanolamine. Figure 5A shows the separation of a sample containing ephedrine and lidocaine, the sample in 5B contains ephedrine, lidocaine, and caffeine. Table 1 and Figures 5B and 6 show that caffeine and lidocaine co-elute under these chromatographic conditions. However, the A_{254}/A_{280} absorbance ratios can be used to identify those peaks that are a mixture of these 2 solutes. Lidocaine and caffeine have very different absorbance ratios (see Table 1) and a comparison of Figures 5A and 5B illustrates the use of this ratio for detecting co-eluting solutes.

Figure 6 shows the chromatogram of a com-

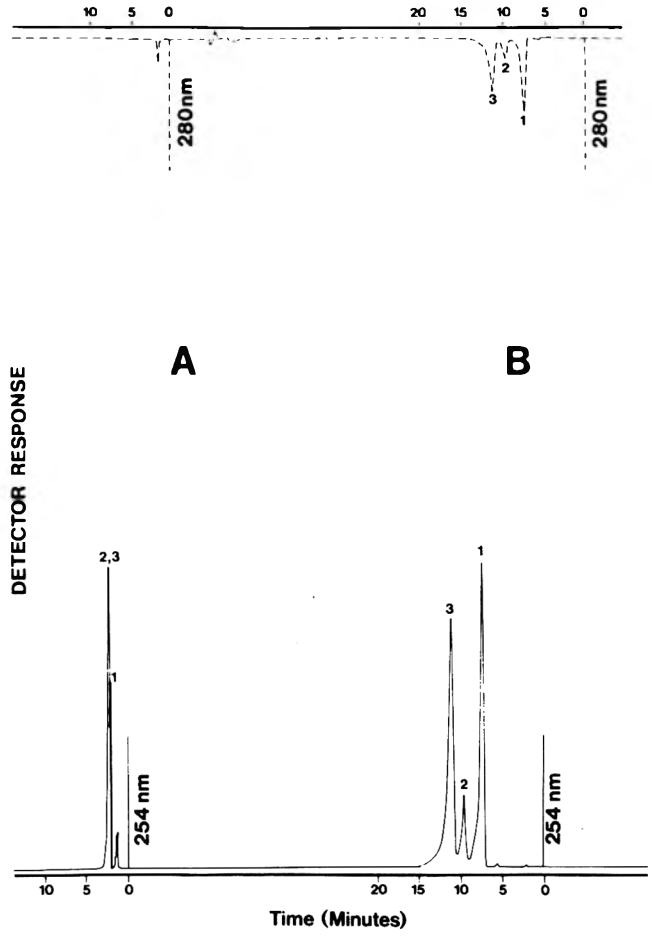

Figure 8. Liquid chromatogram of amines found in cocaine look-alike samples. A, underderivatized amines. Peaks: 1, phenylpropanolamine; 2, ephedrine; 3, pseudoephedrine. Mobile phase, Solvent a. B, derivatized amines. Peaks: 1, ephedrine-PIT; 2, phenylpropanolamine-PIT; 3, pseudoephedrine-PIT. Mobile phase, Solvent b.

monly encountered cocaine look-alike preparation containing pseudoephedrine, lidocaine, tetracaine, and caffeine. Figure 7 illustrates another recently analyzed forensic sample which is a mixture of procaine, tetracaine, and phenylpropanolamine. The amines most often encountered in the cocaine look-alike products are ephedrine, pseudoephedrine, and phenylpropanolamine. These protonated amines elute very early in the chromatographic system used for the analysis of cocaine samples (see Figure 8A).

For those samples suspected of containing the quick-eluting amines, a derivatization procedure is used to identify the specific amine. These amines produce strong UV-absorbing products when treated when phenylisothiocyanate (5). The chromatograms in Figure 8 illustrate the advantages of this derivatization method. Figure 8A shows the results of injecting a solution containing 25 μg each of ephedrine, pseudoephedrine, and phenylpropanolamine hydrochlorides. Extraction of the same quantity of these amines into methylene chloride followed by derivatization with phenylisothiocyanate produced the chromatogram in Figure 8B. The sensitivity of the UV detector was reduced from 0.2 to 2.0 AUFS to keep the peaks on-scale. This separation of the PIT derivatives requires different mobile phase conditions (Solvent b) than the cocaine analysis, but readily distinguishes the 3 commonly encountered amines. Most local anesthetics are tertiary amines and do not form stable PIT derivatives. The use of this method

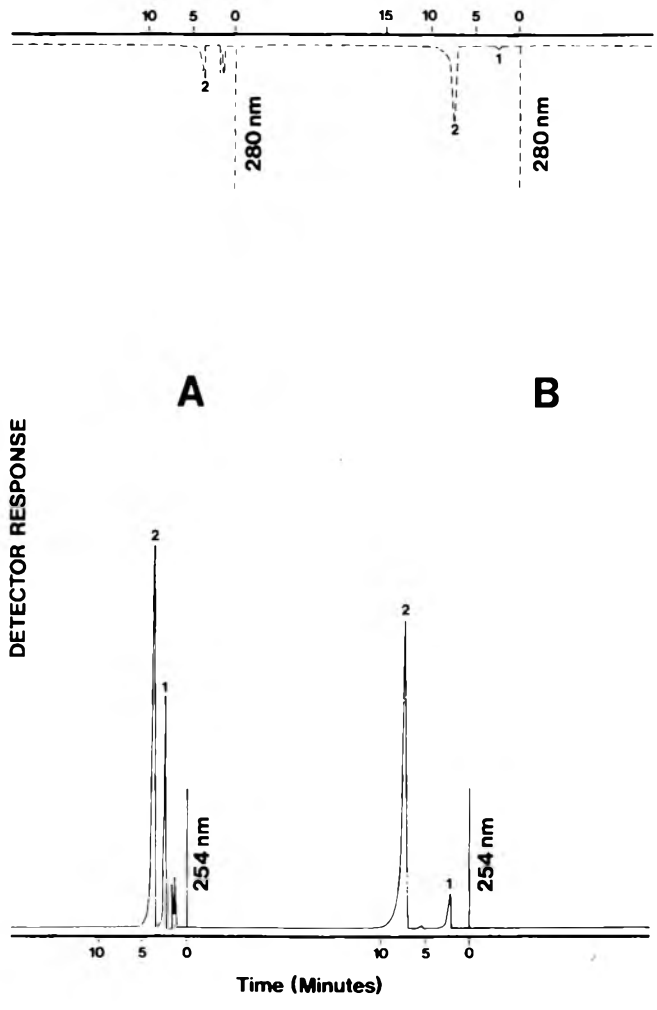

Figure 9. Analysis of cocaine look-alike sample, using PIT derivatization. A, underivatized sample. Peaks: 1, ephedrine; 2, lidocaine. Mobile phase, Solvent a. B, derivatized sample. Peaks: 1, lidocaine; 2, ephedrine-PIT. Mobile phase, Solvent b.

in the analysis of a cocaine look-alike sample is shown in Figure 9. Figures 9A and 9B show the chromatography of the sample before and after derivatization, respectively. All local anesthetics studied with the exception of dibucaine elute before the PIT-amines in solvent system b.

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Simple Colorimetric Method for Determination of Pyridoxine Hydrochloride (Vitamin B_6) in Pharmaceuticals

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A simple colorimetric method is described for the determination of pyridoxine hydrochloride (vitamin B_6). The method is based on the measurement of an orange species formed when pyridoxine hydrochloride is treated with diazotized dapsone and sulfanilamide in a mixture of trichloroacetic acid and sulfuric acid at room temperature, followed by treatment with an aqueous solution of sodium carbonate. Compounds such as thiamine hydrochloride, cyanocobalamin, and common excipients such as starch and talc which are present in various formulations with pyridoxine hydrochloride do not interfere in the reaction. Statistical validation showed that the method was highly precise and accurate. Results agree well with those obtained by other methods reported in the literature.

Pyridoxine hydrochloride, Py-HCl (5-hydroxy-6-methyl-3,4-pyridinedimethanol hydrochloride) or vitamin B_6 hydrochloride, is an enzyme co-factor vitamin. The official compendia (1, 2) describe a method for nonaqueous titration with perchloric acid, using crystal violet as an indicator. Various titrimetric (3), spectrophotometric (4, 5), electrophoretic (6), and chromatographic (7, 8) methods have been described for determining Py-HCl in biological samples and in mixtures of vitamins.

In the present communication, a simple photometric method is described for determining Py-HCl in plain formulations and also in the presence of vitamins B_1 and B_{12} and common excipients. The method is much simpler and faster than those reported in the literature.

METHOD

Reagents

(a) *Pyridoxine hydrochloride standard solution.*—Dissolve 25 mg accurately weighed Py-HCl in 100 mL water in volumetric flask, with shaking.

(b) *Dapsone and sulfanilamide working solutions.*—(1) *Dapsone.*—Dissolve 100 mg dapsone in 100 mL 95% ethanol. (2) *Sulfanilamide.*—Dissolve 100 mg sulfanilamide in 20 mL 95% ethanol, and dilute solution to 100 mL with water.

(c) *Acid mixture.*—15% trichloroacetic acid–H_2SO_4–water (86 + 20 + 94).

(d) *Sodium nitrite solution.*—0.1 % aqueous.

(e) *Sodium carbonate solution.*—4.0% aqueous.

Preparation of Assay Solution

Tablets.—Powder 20 tablets and accurately weigh quantity equivalent to 25 mg Py-HCl. Dissolve in about 50 mL water. Filter through Whatman No. 1 paper. Collect filtrate and washings in 100 mL standard flask and dilute to 100 mL with water.

Injections.—From thoroughly shaken solution from injection vial, transfer volume equivalent to 25 mg Py-HCl into 100 mL standard flask and dilute to 100 mL with water.

Preparation of Standard Curve

Dilute Py-HCl standard solution to give concentrations of 25–175 μg/mL. Develop color and read absorbance of reaction product, as described under *Assay*. Plot absorbance vs Py-HCl concentration to give straight line passing through origin. Beer's law is obeyed between 1 and 7 μg/mL. Molar absorptivities are 2.1×10^4 and 2.6×10^4 L^{-1} $mole^{-1}$ cm^{-1} for sulfanilamide and dapsone, respectively.

Assay

Sulfanilamide.—Pipet 0.25 mL sulfanilamide working solution into each of series of 25 mL graduated flasks. Add 1 mL acid mixture and 1 mL sodium nitrite solution. Let flasks stand 3 min. To individual flasks add 0.1–0.7 mL Py-HCl and 3 mL sodium carbonate solution, with constant shaking. Simultaneously prepare reagent blank. Dilute mixed solutions to volume with water after 3 min, and measure absorbances at 440 nm against reagent blank. Determine concentration in test solution from standard curve, using same procedure detailed earlier.

Dapsone.—Pipet 0.5 mL dapsone working solution into each of series of 25 mL graduated flasks. Add 1 mL acid mixture and 1 mL sodium nitrite solution to each flask. Let flasks stand 3 min, then add 2 mL 95% ethanol to each flask. Add 0.1–0.7 mL Py-HCl and 3 mL sodium carbonate solution to individual flasks with constant shaking. Simultaneously prepare reagent blank.

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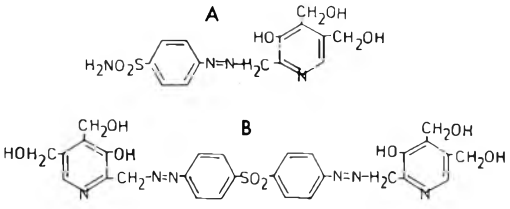

Figure 1. Probable structure for reaction of pyridoxine HCl with diazotized sulfanilamide (A) or dapsone (B).

Dilute mixed solutions to volume with water after 3 min, and measure absorbances at 465 nm against reagent blank. Determine concentration in test solution from standard curve, using same procedure detailed earlier.

Stoichiometry of Reaction Products

The reactions reported make use of diazotization of sulfanilamide and dapsone followed by coupling with vitamin B_6 for the color development. The reactions are well known normal dye formation, only their applications in this method are important. The products were separated and analyzed. Probable structures determined from infrared and nuclear magnetic resonance spectral studies are given in Figure 1.

Recovery Experiment

To study reliability and recovery of the proposed method, we used a method of standard addition. A fixed weight of sample was taken and standard drug was added at 3 different levels. Each level was repeated 7 times. The total amount of the drug was determined by the proposed method.

Percent recovery was calculated as follows:

$$\% \text{ recovery} = [N \Sigma XY - (\Sigma X)(\Sigma Y) \times 100]/(N \Sigma X^2 - (\Sigma X)^2)$$

where X = amount of drug added/g sample; Y = amount of drug found/g sample; N = total number of observations.

Results and Discussion

Pyridoxine hydrochloride reacts with diazotized dapsone and sulfanilamide under the operative reaction conditions to yield a colored product that undergoes a bathochromic shift (465 nm and 440 nm, respectively) when it is made alkaline with sodium carbonate. The orange color thus developed has been successfully used to determine pyridoxine hydrochloride in formulations.

The proposed method has distinct advantages over similar methods in the literature for determining pyridoxine hydrochloride in combinations with thiamine hydrochloride and cyanocobalamin. The published spectrophotometric methods specify techniques such as paper chromatography, electrophoresis, or thin layer chromatography to separate Py-HCl before its determination. The present method is direct, fast, simple, sensitive, and accurate. The diazotization and coupling reactions are carried out at room temperature, addition of ammonium sulfamate to remove excess nitrite ions is unnecessary, and color development and absorbance measurement are complete within 20 min. The molar absorptivity values indicate the high

Table 1. Determination of pyridoxine hydrochloride by the proposed method, for reaction with sulfanilamide or dapsone

Product	Amt declared, mg	Found, mg		% Rec. by addition method		Std dev		Coeff. of var., %	
		Sulfanilamide	Dapsone	Sulfanilamide	Dapsone	Sulfanilamide	Dapsone	Sulfanilamide	Dapsone
Tablet:									
Pyridoxine HCl	40	41.1	39.2	102.9	98.09	0.079	0.10	1.59	2.16
Tablet:									
Pyridoxine HCl	20	—	19.41	—	97.04	—	0.094	—	1.96
Thiamine HCl	100								
Cyanocobalamin	50[a]								
Injection:									
Pyridoxine HCl	10	10.062	—	100.6	—	0.095	—	1.85	—
Thiamine HCl	33								
Cyanocobalamin	333[a]								
Injection:									
Pyridoxine HCl	33	32.6	32.4	98.8	98.25	0.074	0.10	1.60	2.23
Thiamine HCl	33								
Cyanocobalamin	333[a]								

[a] Amount declared in μg.

Table 2. Recovery (%) of pyridoxine HCl added to formulations, for reaction with sulfanilamide or dapsone

Product	Amt declared, mg	Sulfanilamide 1st level	Sulfanilamide 2nd level	Sulfanilamide 3rd level	Dapsone 1st level	Dapsone 2nd level	Dapsone 3rd level	% Declared, IP/USP
Tablet:								
Pyridoxine HCl	40	102.7[a]	103.4	102.59	97.8	98.4	98.07	95–115
Tablet:								
Pyridoxine HCl	20	—	—	—	97.23	96.97	97.02	—
Thiamine HCl	100							
Cyanocobalamin	50[b]							
Injection:								
Pyridoxine HCl	10	101.31	99.69	100.86	—	—	—	—
Thiamine HCl	33							
Cyanocobalamin	333[b]							
Injection:								
Pyridoxine HCl	33	98.2	98.8	99.4	97.83	98.16	98.75	—
Thiamine HCl	33							
Cyanocobalamin	333[b]							

[a] Amount declared in μg.
[b] Each value is the average for 7 determinations.

sensitivity of the method. Results by the proposed method are in excellent agreement with those obtained by pharmacopeial methods.

Percent recoveries obtained from the addition method are in the range 97–103%, indicating lack of interference by other ingredients and excipients in the determination (Table 1). The method is also reproducible as shown by results in Table 2. Therefore, we recommend this method in the routine quality control analysis of Py-HCl in formulations containing vitamins B_1 and B_{12}.

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X-Ray Powder Diffraction Data for Nine Anthelmintics

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X-ray powder diffraction data for identifying 9 anthelmintics have been obtained by diffractometer and Debye-Scherrer camera techniques. The data are tabulated in terms of the lattice spacings and the relative intensities of the lines. Patterns using 3 different X-ray wavelengths with the camera method are compared with each other and with the diffractometer patterns.

X-ray diffraction has been used to characterize 9 anthelmintics in terms of values of d (in Å), the interplanar spacing, and I/I_1, the relative intensities of the lines, based on the strongest lines as 100, according to the system of the *Powder Diffraction File* (1). The data recorded here were not available in the *Powder Diffraction File*. Results from the diffractometer traces (CuKα radiation) were compared with microdensitometer measurements of Debye-Scherrer camera films using 3 wavelengths (CuKα, CoKα, and CrKα).

METHOD

Details of sample preparation, exposures, and measurements of line positions and intensities have been given in a previous paper (2) and are not repeated here. The anthelmintics examined were hexylresorcinol, mebendazole, morantel tartrate, niclosamide, niridazole, parbendazole, thiabendazole, tetramisole hydrochloride, and levamisole hydrochloride. Melting points of all compounds agreed with those quoted in the literature.

Results and Discussion

Tables 1–9 show the data for the 9 anthelmintics examined. Diffractometer results (CuKα radiation) are averages from the McCreery and Byström-Asklund methods of sample loading discussed previously (2), with intensities (I/I_1) relative to the strongest line (I_1) of a pattern as 100 calculated from measurements of peak height above background on the traces. The relative intensities in the camera results (CuKα, CoKα, and CrKα radiation) were derived from microdensitometer traces, although dense backgrounds, which are not uncommon with organic materials, made measurements difficult for some films. The 3 strongest lines for each substance would be used for preliminary identification in the *Powder Diffraction File* method, and are therefore given in boldface type in the tables.

In general there was good agreement among the interplanar spacings (d in Å) for the 4 sets of results for a given compound, but the differences recorded, which were not greater than 0.06Å, do illustrate the variations to be anticipated when powder data are used for identification. As before, the best resolution of the lines was obtained by the diffractometer method. With the photographic technique, the best was found with CrKα radiation, which has the longest wavelength of the 3 employed.

With the exceptions of tetramisole hydrochloride (Table 8) and levamisole hydrochloride (Table 9), where the second strongest line shown in the diffractometer traces differed from that in the films, there was agreement in the 4 sets of results on the identity of the 3 strongest lines. The diffractometer patterns for the 2 compounds mentioned were less satisfactory than for the others in that different sample loading methods led to greater than usual variations in line resolution and intensity, probably because the materials tended to absorb water from the atmosphere.

There was agreement among the 4 sets on the location of the strongest line ($I/I_1 = 100$) except for morantel tartrate (Table 3), where the strongest in the diffractometer pattern was recorded as the third line with relative intensity 68–81 in the camera patterns. Preferred orientation probably affected the diffractometer results here in spite of careful loading of the samples, because the second and third most intense lines and all the others were relatively much weaker than the corresponding lines in the films. In fact, most compounds showed similar discrepancies in varying degrees between camera and diffractometer intensities, particularly for the larger interplanar spacings, and these effects were more marked than in the 2-imidazolines examined previously (2). Users of the *Powder Diffraction File* are accustomed to such variations when they

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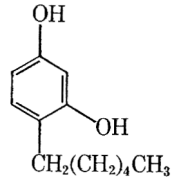
Table 1. X-ray diffraction data for hexylresorcinol [a]



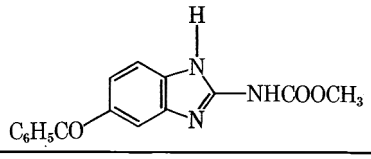
$C_{12}H_{18}O_2$

CAS-136-77-6

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1
21.0	**100**	**21.4**	**100**	**21.4**	**100**	**21.4**	**100**
10.7	1	—	—	—	—	—	—
7.05	2	7.04	5	7.02	1	—	—
5.54	5	5.51	28	5.53	10	5.52	13
5.28	9	5.27	22	5.23	12	5.25	8
5.14	1	—	—	—	—	—	—
4.96	1	4.94	6	—	—	—	—
4.71	**12**	**4.70**	**61**	**4.70**	**32**	**4.69**	**33**
4.59	1	—	—	—	—	—	—
4.35	1	—	—	—	—	—	—
4.31	2	4.29	18	4.30	10	4.29	9
4.23	1	—	—	—	—	—	—
4.08	4	**4.07**	33	4.07	16	4.07	18
3.87	1	3.84	5	—	—	—	—
3.71	**13**	—	—	—	—	—	—
3.66	10	**3.66**	**69**	**3.66**	**54**	**3.66**	**49**
3.53	4	—	—	—	—	—	—
3.49	3	3.48	21	3.48	10	3.48	10
3.38	2	3.37	12	3.37	6	3.37	6
3.14	1	3.13	7	3.13	3	3.13	3
3.03	1	3.03	2	3.02	2	3.03	2
2.776	1	2.763	5	2.765	2	2.758	4
2.652	2	2.646	5	2.643	3	2.643	2
2.589	1	—	—	—	—	2.573	2
2.356	2	2.354	7	2.353	4	2.348	6
2.127	1	2.121	7	2.124	3	2.123	2
2.090	1	2.082	3	2.081	3	2.080	2

[a] The 3 most intense lines are shown in boldface type.

Table 2. X-ray diffraction data for mebendazole [a]



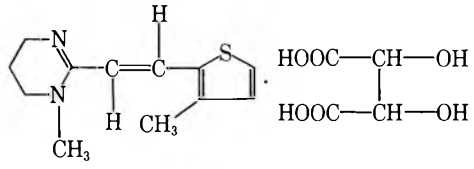
$C_{16}H_{13}N_3O_3$

CAS-31431-39-7

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1
18.0	**100**	**17.8**	**100**	**17.5**	**100**	**17.9**	**100**
7.50	6	—	—	—	—	—	—
7.20	17	7.17	43	7.15	37	7.15	13
6.71	3	6.63	7	6.65	10	6.63	3
6.09	4	6.05	16	6.03	11	6.02	5
5.47	33	5.45	63	5.44	55	5.43	23
4.90	33	4.85	75	4.85	67	4.85	27
4.62	32	—	—	—	—	4.59	19
4.51	**72**	**4.48**	**89**	**4.47**	**87**	**4.46**	**40**
4.15	24	4.12	47	4.12	41	4.11	17
3.75	9	3.72	21	3.72	20	3.73	8
3.60	49	3.59	78	3.58	76	3.58	30
3.54	22	—	—	—	—	3.51	16
3.34	**73**	**3.31**	**91**	**3.31**	**97**	**3.31**	**44**
3.07	25	3.06	54	3.05	48	3.05	21
2.858	3	2.834	10	2.836	7	2.840	5
—	—	2.757	6	2.758	4	2.764	3
2.681	3	2.667	15	2.668	13	2.662	4
2.600	5	—	—	—	—		
2.585	5	2.580	21	2.574	15	—	—
2.515	2	2.503	9	2.501	7	—	—
		2.381	7	2.380	7	—	—
		2.177	10	2.176	9		
		2.115	12	2.112	11		

[a] The 3 most intense lines are shown in boldface type.

Table 3. X-ray diffraction data for morantel tartrate [a]



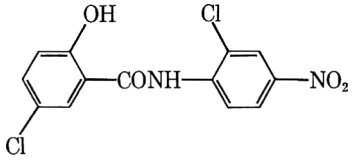
$C_{12}H_{16}N_2S \cdot C_4H_6O_6$

CAS-20574-50-9 parent
CAS-26155-31-7 tartrate

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1
12.4	27	—	—	12.4	21	12.4	20
—	—			—	—	11.1	16
10.4	11	—	—	10.3	22	10.3	18
9.51	2	—	—	9.40	14	—	—
7.56	8	7.52	20	7.52	19	7.54	23
7.11	1	—	—	7.09	8	—	—
6.46	1	6.50	21	6.49	12	—	—
5.89	17	5.95	10	5.97	14	—	—
5.61	2	5.59	20	5.59	15	—	—
5.19	**100**	**5.18**	**81**	**5.17**	**76**	**5.18**	**68**
4.87	5	4.88	31	4.87	36	4.86	25
4.75	11	4.73	46	4.74	48	4.73	27
4.65	4	—	—	—	—	4.62	16
4.43	4	—	—	—	—	—	—
4.30	5	4.29	12	4.29	11	4.28	14
4.11	1	—	—	—	—	—	—
4.03	12	4.02	28	4.01	33	4.01	32
3.82	5	—	—	3.80	25	3.79	16
3.74	**19**	**3.74**	**100**	**3.73**	**100**	**3.72**	**100**
3.66	5	—	—	3.66	9	3.64	23
3.59	**18**	**3.57**	**88**	**3.56**	**85**	**3.57**	**89**
3.47	1	—	—	—	—	—	—
3.38	9	3.38	30	3.38	38	3.37	27
3.27	4	3.27	26	3.26	26	3.25	23
3.21	5	3.20	22	3.20	24	3.20	16
3.16	4	3.15	22	3.15	25	3.14	25
3.09	5	—	—	3.07	6	—	—
3.05	3	3.06	7			—	—
2.979	5	2.962	15	2.973	15	2.964	14
2.936	4	—	—	2.904	7	—	—
2.885	2	—	—			—	—
2.844	1	—	—	—	—	—	—
2.780	2	—	—	—	—	—	—
2.730	1	—	—	—	—	—	—
2.691	1	2.681	5	2.677	5	—	—
2.600	2	2.591	6	—	—	—	—
2.515	3	3.507	22	2.505	24	2.505	14
2.468	3	2.441	12	2.442	12	2.438	13
2.392	4	2.389	11	—	—	2.390	12
2.338	2	2.330	12	2.327	12	2.327	10
		2.213	7	—	—		
		2.075	4	—	—		
		2.003	8	2.000	8		

[a] The 3 most intense lines are shown in boldface type.

Table 4. X-ray diffraction data for niclosamide [a]



$C_{13}H_8Cl_2N_2O_4$

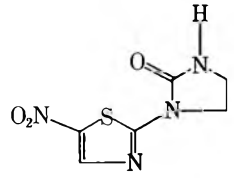
CAS-50-65-7

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1
13.5	3	12.4	11	13.4	15	13.4	35
11.6	8	10.9	17	11.5	11	11.5	22
9.41	27	9.38	28	9.40	29	9.38	52
7.66	36	7.63	42	7.64	36	7.64	65
6.94	14	—	—	—	—	—	—
6.81	32	6.80	44	6.79	41	6.78	26
6.46	**37**	**6.43**	**50**	**6.43**	**42**	**6.44**	**57**
5.26	14	5.22	19	5.23	16	5.26	35
5.16	6	—	—	—	—	—	—
4.77	5	4.74	15	4.76	9	—	—
4.48	26	4.47	36	4.48	33	4.45	26
4.00	17	3.99	21	3.99	20	—	—
3.93	12	—	—	—	—	—	—
3.82	36	3.81	39	3.81	38	3.80	52
3.74	8	—	—	—	—	—	—
3.58	19	3.58	28	3.57	26	3.58	30
3.47	**100**	**3.46**	**100**	**3.47**	**100**	**3.47**	**100**
3.39	48sh [b]	3.39	72sh	3.39	69sh	3.39	57sh
3.34	**81**	**3.33**	**92**	**3.32**	**83**	**3.33**	**74**
3.28	49sh	3.27	75sh	3.26	79sh	3.27	70sh
3.20	5	3.20	11	—	—		
3.18	5	—	—	3.18	11		
3.12	5			3.11	7		
3.08	5	3.10	8	3.07	9		
3.06	5	3.05	8	3.05	5		
2.988	4	—	—	2.973	6		
2.867	5	—	—	2.849	6		
2.805	6	—	—	2.801	11		
2.683	5	2.671	11				
2.652	7	—	—				

[a] The 3 most intense lines are shown in boldface type.

[b] sh = shoulder.

Table 5. X-ray diffraction data for niridazole [a]



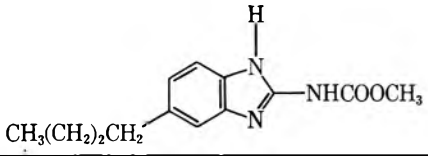
$C_6H_6N_4O_3S$

CAS-61-57-4

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1
9.88	7	—	—	—	—	—	
6.17	**37**	**6.15**	**85**	**6.15**	**76**	**6.13**	**36**
5.45	15	5.43	54	5.44	47	5.43	21
4.93	22	4.92	17	4.91	12	4.92	9
4.61	15	4.60	55	4.60	52	4.59	22
4.48	**23**	**4.48**	**68**	**4.47**	**69**	**4.47**	**27**
4.17	23	4.16	67	4.16	61	4.16	27
3.93	2	3.92	8	3.91	9	—	—
3.36	11	3.36	28	3.36	23	3.36	8
3.27	**100**	**3.27**	**100**	**3.27**	**100**	**3.26**	**100**
2.926	2	—	—	—	—	—	—
2.885	9	2.880	34	2.883	35	2.878	15
2.780	2	2.780	8	2.781	7	2.778	5
2.722	1	2.715	6	2.720	6	2.717	9
2.671	11	2.667	27	2.668	25	2.662	11
2.501	2	2.501	6	2.496	5	—	—
2.462	5	2.454	4	2.445	5	—	—
2.401	2	2.398	8	2.395	7	—	—
2.338	3	2.340	15	2.338	13	2.332	5
2.304	14	2.300	32	2.299	29	2.300	14
2.176	5	2.171	17	2.168	16	2.165	10
2.146	1	—	—	—	—	—	—
2.113	1	2.106	5	2.105	6	—	—
2.054	4	—	—	—	—	—	—
2.040	4	2.045	22	2.041	23	2.041	13

[a] The 3 most intense lines are shown in boldface type.

Table 6. X-ray diffraction data for parbendazole [a]



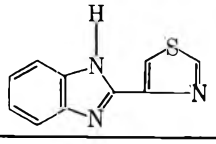
$C_{13}H_{17}N_3O_2$

CAS-14255-87-9

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1
13.1	**100**	**12.9**	**100**	**12.9**	**100**	**13.0**	**100**
9.77	11	9.72	51	9.69	63	9.67	36
8.63	1	8.63	4	8.55	6	8.52	3
6.51	4	6.50	8	6.49	10	6.49	5
6.01	3	5.93	8	5.93	11	5.91	6
5.00	2	—	—	—	—	—	—
4.87	5	4.84	18	4.84	28	4.85	13
4.48	7	4.48	25	4.48	32	4.47	15
4.29	**11**	**4.27**	**32**	**4.28**	**41**	**4.27**	**18**
4.10	10	4.08	31	4.08	40	4.08	17
3.95	6	3.94	19	3.93	26	3.94	10
3.69	7	—	—	3.69	27	—	—
3.67	6	—	—	—	—	—	—
3.61	8	3.62	25	—	—	3.63	5
3.58	7	—	—	3.57	29	—	—
3.34	10	3.34	27	3.34	33	3.34	15
3.15	1	—	—	—	—	—	—
3.11	1	—	—	—	—	—	—
3.08	1	—	—	—	—	—	—
3.06	1	—	—	—	—	—	—
2.763	1	2.761	6	2.758	6	2.750	4
—	—	2.470	4	2.471	5	2.459	3
2.380	1	2.412	4	2.409	3	2.409	3
2.362	1	—	—	—	—	—	—
2.341	1	2.262	2	2.263	4	—	—
2.032	1	—	—	2.075	2	—	—
2.025	1	—	—	2.009	2	—	—

[a] The 3 most intense lines are shown in boldface type.

Table 7. X-ray diffraction data for thiabendazole [a]



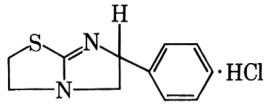
$C_{10}H_7N_3S$

CAS-148-79-8

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1
6.81	8	6.76	17	6.76	17	6.76	13
5.64	4	—	—	—	—	—	—
5.56	7	5.57	18	5.57	14	5.57	11
5.07	**43**	**5.03**	**68**	**5.04**	**75**	**5.03**	**63**
4.23	**100**	**4.21**	**100**	**4.21**	**100**	**4.21**	**100**
—	—	4.02	3	4.01	5	—	—
3.72	14	3.71	26	3.71	27	3.70	20
3.39	**43**	**3.38**	**51**	**3.38**	**51**	**3.38**	**43**
3.27	2	3.26	5	3.26	7	3.25	7
—	—	3.11	3	3.10	4	—	—
2.814	10	2.803	19	2.806	21	2.808	14
2.695	2	2.681	6	2.685	7	—	—
—	—	2.629	3	2.630	7	—	—
2.525	3	2.517	12	2.515	12	2.519	8
—	—	2.419	3	2.418	17	—	—
2.327	3	2.321	9	2.316	21	2.311	7
—	—	2.253	4	2.252	5		
—	—	2.137	5	2.132	4		
2.062	2	2.055	7	2.056	7		
2.025	1	2.022	4	2.000	5		

[a] The 3 most intense lines are shown in boldface type.

Table 8. X-ray diffraction data for (±) tetramisole hydrochloride [a]



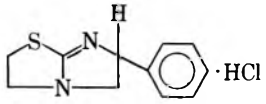
$C_{11}H_{12}N_2S{\cdot}HCl$

CAS-(±)-5036-02-2 parent
CAS-(±)-5086-74-8 HCl

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1	*d*(Å)	I/I_1
11.6	5	11.4	32	11.4	29	—	—
8.27	9	8.29	49	8.29	45	8.26	35
7.50	**22**	7.50	27	7.47	28	7.49	30
6.13	2	6.11	20	6.10	20	—	—
5.61	3	5.64	50	5.62	46	5.61	40
5.45	3	5.43	67	5.44	58	5.43	60
5.23	1	5.22	10	5.21	12	—	—
4.70	9	**4.66**	**95**	**4.66**	**96**	**4.66**	**95**
4.46	2	4.45	32	4.45	38	4.43	35
4.27	3	4.27	42	4.26	40	4.24	30
4.14	5	4.13	30	4.13	27	4.12	20
3.97	6	3.94	55	3.95	56	3.95	70
3.92	3					—	—
3.75	**100**	**3.74**	**100**	**3.75**	**100**	**3.74**	**100**
3.66	8	3.65	57	3.65	57	3.64	55
3.47	2	3.46	24	3.47	18	3.46	35
3.33	**12**	**3.32**	**92**	**3.33**	**81**	**3.32**	**90**
3.20	2	3.20	15	3.20	16	—	—
3.05	2	3.05	10	3.06	12	—	—
2.885	3	2.887	37	2.883	39	2.873	30
2.818	5	2.816	64	2.817	63	2.813	15
2.759	2	2.743	25	2.734	28	2.728	30
2.730	2	2.660	12	2.660	11		
2.611	3	2.607	25	2.609	23	2.600	20
2.532	3	2.536	25	2.534	28	2.527	40
2.491	5	2.490	8	2.488	6	—	—
—	—	2.377	17	2.378	19	—	—
2.225	2	2.225	17	2.228	20	—	—

[a] The 3 most intense lines are shown in boldface type.

Table 9. X-ray diffraction data for (−) levamisole hydrochloride [a]



(−) $C_{11}H_{12}N_2S$·HCl

CAS-14769-73-4 parent
CAS-16595-80-5 HCl

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1
7.41	27	7.34	39	7.34	**30**	7.34	27
6.51	41	—	—	—	—	—	—
6.44	**42**	6.41	32	6.38	24	6.37	27
5.97	10	—	—	—	—	—	—
5.54	16	**5.50**	**80**	**5.49**	**74**	**5.49**	**73**
5.44	5	—	—	—	—	—	—
5.10	11	5.06	29	5.05	28	5.04	33
4.90	6	4.82	4	4.83	4	—	—
—	—	4.62	**7**	4.61	7	—	—
4.37	**56**	**4.34**	**95**	**4.34**	**99**	**4.34**	**97**
4.22	15	4.19	19	4.19	25	4.17	20
3.93	35	3.90	22	3.91	21	3.89	30
3.80	18	3.78	45	3.78	38		
3.74	21	—	—	—	—	3.76	40
3.69	**100**						
3.65	18	**3.67**	**100**	**3.67**	**100**	**3.67**	**100**
3.55	5	—	—	—	—	—	—
3.51	2	3.52	5	—	—	—	—
3.41	10	3.39	9	3.39	10	3.39	27
3.27	28	3.26	38	3.26	35	3.25	40
3.24	42						
3.17	22	—	—	—	—	—	—
3.13	23	3.12	49	3.12	43	3.12	33
3.05	25	3.04	37	3.03	35	3.03	47
2.969	3	—	—	—	—	—	—
2.849	15	—	—	—	—	—	—
2.823	12	2.823	32	2.827	25	2.825	27
2.805	11	—	—	—	—	—	—
2.698	3	—	—	2.685	13	2.699	33
2.660	13	2.648	29	2.646	25	2.645	27
2.592	1	—	—	—	—	—	—
2.557	10	—	—	—	—	—	—
2.543	6	2.529	12	2.527	13	2.530	20
2.498	1	—	—	—	—	—	—
2.449	32	2.439	19	2.441	16	2.438	27
2.417	2	—	—	—	—	—	—
2.350	1	—	—	—	—	—	—
2.327	2	2.312	5	2.316	6	—	—
2.293	3	—	—	—	—	—	—
2.254	8	2.254	12	2.255	9	2.246	20
2.238	2	—	—	—	—	—	—
2.181	4	2.177	9	2.178	4	2.171	20
2.161	2	—	—	—	—	—	—
2.137	4	—	—	—	—	—	—
2.120	4	2.123	6	2.128	6		
2.045	4	—	—	—	—		
2.034	3	2.032	12	2.036	8		

[a] The 3 most intense lines are shown in boldface type.

compare film data with file data from diffractometers and vice versa.

Data for tetramisole hydrochloride and levamisole hydrochloride (Tables 8 and 9) are of particular interest because the first is the racemic- and the second the levo-rotatory form of the same compound, so that identical X-ray diffraction patterns would be expected. It has already been mentioned that the results for these samples were not as consistent as for others, but the patterns were certainly different, and it appeared that the 2 had crystallized with different structures. An investigation of polymorphism in the compound would be required to elucidate the problem.

Conclusion

X-ray powder diffraction could be used to identify the 9 anthelmintics examined, although the apparent distinction between tetramisole hydrochloride and levamisole hydrochloride should be treated with caution. The diffractometer method or the camera method using CrKα radiation would be acceptable unless many lines from other substances were present. Discrepancies between diffractometer and camera data are no greater than those often noticed when working with the *Powder Diffraction File.*

REFERENCES

(1) *Powder Diffraction File,* Joint Committee on Powder Diffraction Standards, Swarthmore, PA

(2) Owen, J. T. R., Koutourellis, J. E., & Underwood, F. A. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1164–1173

Determination of Gentamicin Sulfate C_{1a}, C_2, and C_1 Components by Ion Pair Liquid Chromatography with Electrochemical Detection

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The 3 major components of gentamicin sulfate (C_{1a}, C_2, and C_1) were separated by a liquid chromatographic method using a reverse phase C_{18} bonded silica gel column and an aqueous ion pair solvent system consisting of 0.015M pentanesulfonic acid sodium salt, 0.2M sodium sulfate, and 0.1% acetic acid. Individual C components as well as several unknown minor constituents were detected by an electrochemical flow cell with a glassy carbon working electrode set at a potential of +1.3 ± 0.1 V vs Ag/AgCl. The analytical response was linear over a concentration range of 16-30 μg. A plot of peak areas vs concentration of C fractions yielded a correlation coefficient of 0.999 or better for the C_{1a}, C_2, and C_1 components of gentamicin sulfate.

Gentamicin, an aminoglycoside antibiotic produced by *Micromonospora purpurea*, was isolated by Weinstein et al. (1) in 1963. Using paper chromatography, Rosselet and coworkers (2) determined that gentamicin consisted of approximately 72% C_1 and 28% C_2. In later work by descending paper chromatography using a chloroform–methanol–ammonium hydroxide solvent system, Weinstein et al. (3) separated the C_1 component into 2 fractions labeled C_1 and C_{1a}. This chromatographic method followed by microbiological assay of the separated fractions was adopted as the Food and Drug Administration (FDA) official method for gentamicin (4, 5). Additional studies revealed several minor constituents in the gentamicin antibiotic (6–9). Byrne et al. (10) reported that 5 components were isolated from the gentamicin C-complex by counter-current distribution: 3 major fractions, C_1, C_2, and C_{1a}, and 2 minor components designated C_{2a} and C_{2b}, which represented approximately 4% of the gentamicin composition. Thomas and Tappin (11) used ion-exchange chromatography with detection by optical rotation to determine gentamicin and found 9 components other than C_1, C_2, and C_{1a}, 4 of which were biologically active. Several analysts (12–15) separated gentamicin by liquid chromatography using a reverse phase system with fluorescence detection after derivatization with *o*-phthalaldehyde (OPA) (12, 14, 15) or dansyl chloride (13). Some of the gentamicin separations on reverse phase columns were performed with ion pair couplings. Perruchon and Caullet have hydrolyzed several aminoglycosides and determined them by differential pulse polarography (16).

The liquid chromatographic fluorescence assay of gentamicin with post-column OPA derivatization has been investigated by the National Center for Antibiotics Analysis (NCAA) as a possible replacement for the FDA official (5) paper chromatographic assay. A report of this work has shown some difficulties with the reagents (W. Walton, Food and Drug Administration, personal communication, 1980). Because a convenient, reliable assay protocol is needed to determine the composition of the marketed gentamicin products, a liquid chromatographic-electrochemical detection (LCED) method has been developed, with separation of the gentamicin components on reverse phase columns with ion pair reagents.

METHOD

Apparatus

(a) *Column.*—30 cm × 3.9 mm id stainless steel column, packed with 10 μm particle size C_{18} reverse phase μBondapak (Waters Associates, Milford, MA). At the end of each day's use, flush column copiously with water and fill with methanol (Burdick and Jackson Laboratories, Muskegon, MI, or equivalent). Once a particular column is deemed satisfactory, it should be dedicated to the gentamicin analysis.

(b) *Liquid chromatograph.*—DuPont Model 850 liquid chromatograph, with a 3-piston pumping system to minimize pulsations, and equipped with manual 20 μL loop injector; electrochemical detector consisting of E611 potentiostat and EA1096 3-electrode flow cell (Metrohm/Brinkmann, Westbury, NY). Working electrode (GCE, glassy carbon electrode), set at oxidizing potential of +1.3 ± 0.1 V vs Ag/AgCl reference, and GCE auxiliary electrode. Current sensitivity from 500 to 3000 nA full scale recorder deflec-

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tion. Use dc mode with full damping for detection. Because of the negative deflection of the anodic signal, connect output leads from the potentiostat in parallel with recorder (Linear Instruments Corp., Irvine, CA) and integrator (Supergrator I, Columbia Scientific Industries, Austin, TX) with the negative and positive signals inverted at the integrator input. Attenuate signal to integrator by ca 90% to accommodate voltage input range.

Reagents

(Use deionized and filtered water throughout.)

(a) *Solvent system (15).*—0.05M pentanesulfonic acid sodium salt (Eastman, Rochester, NY), 0.2M sodium sulfate (Merck, Rahway, NJ), and 0.1% acetic acid (Mallinckrodt Chemical Co., St. Louis, MO), all as received. Adjust mobile phase to maximize resolution for different columns by altering amounts of pentanesulfonic acid sodium salt and sodium sulfate as described by Anhalt et al. (15). Filter mobile phase and deaerate by vacuum draw-off. Flow rate may be varied from 1.0 to 1.25 mL/min.

(b) *Gentamicin sulfate.*—Samples used in this study were manufactured by Schering Corp., Bloomfield, NJ, and Pierrel A.p.A.m. Milan, Italy. Standards were USP Reference Standard Gentamicin; potency, 643 μg/mg.

Determination

Dilute sample and standard solutions to ca 2.5 mg/mL in water, unless indicated otherwise. Dilute individual C fractions to ca 1.0 mg/mL. Inject 20 μL each of standard and sample and obtain chromatogram, using flow rate of 1.0 mL/min. Elution order is C_{1a}, C_2, C_1. Calculate gentamicin components as follows: Prepare a calibration curve of the peak areas vs concentration for the individual C_{1a}, C_2, and C_1 fractions over the range 16–30 μg. Correlate the peak areas of the fractions in the standard to the calibration curve of the respective "C" fraction to ascertain the amount of each major component in the standard. Total these values and determine percent by weight of each fraction by comparing its contribution to this total.

Results

The proposed method was validated by comparing the results of the LCED analysis of the gentamicin standard to those obtained by the official method. For this purpose, purified gentamicin C fractions (C_{1a}, C_2, and C_1) were obtained from the Schering Corp. and the composition of the standard was determined by LCED as described above. Results are shown in Table 1.

Table 1. Determination of USP Reference Standard gentamicin sulfate by LCED method (potency 643 μg/mg) [a]

Day	Gentamicin sulfate, mg/mL	Gentamicin components, % by wt		
		C_{1a}	C_2	C_1
1	2.72	24.2	38.5	37.4
	2.96	24.1	38.0	37.9
2	2.962	23.4	39.1	37.4
	2.63	23.8	38.7	37.6
Mean		23.9	38.6	37.6
SD		1.5	1.2	0.6

[a] Composition of the standard by paper chromatography: C_{1a}, 27.6%; C_2, 39.5%; C_1, 32.9%. See footnote, Table 3.

The agreement found between the 2 methods for the composition of the standard was considered to be satisfactory in view of the number of variables, i.e., 4 different standards, and the number of assays required to obtain the results. As a consequence of this validation study, it was concluded that a gentamicin sample with a known fractional composition, such as the USP standard, can be used as a reference material for the LCED analysis.

Discussion

Electrochemical Response.—Previous work has demonstrated that amines, thiols, and hydroxy groups are easily oxidized on a glassy carbon electrode (M. Hayden and T. Getek, unpublished data). Gentamicin has several amino and hydroxy substituents and these are the probable sources for its electrochemical activity. An increase in the positive potential augmented the current signal, but the baseline deteriorated because of the debilitating effects of the large positive voltages on the glassy carbon. An optimal operating potential was needed that would give a steady baseline and good signal response vs concentration. A manual voltammogram of gentamicin (Figure 1) was obtained by measuring the peak height in nA of the C_{1a} fraction at several voltages. A potential of +1.3 V gave an adequate signal response and produced a constant baseline.

A significant change in the electrochemical response was correlated to the concentration of sodium sulfate. A change from 0.2M to 0.3M reduced the peak height by approximately 40%. Further increases in the concentrations did not

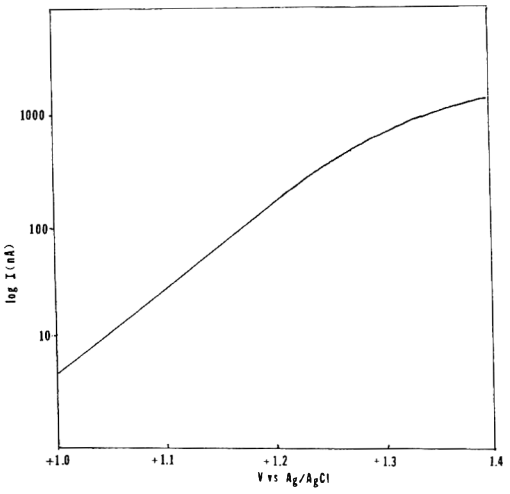

Figure 1. Log current response (nA) vs potential against Ag/AgCl reference. Flow rate 0.8 mL/min; mobile phase consisted of 0.02M pentanesulfonic acid sodium salt, 0.2M Na_2SO_4, and 0.1% acetic acid diluted to 1 L with 3% aqueous methanol.

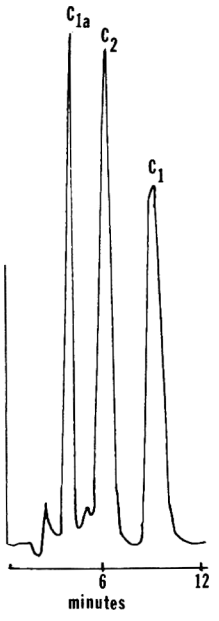

Figure 2. LCED chromatogram of gentamicin sulfate (3.3 mg/mL). Flow rate 1.0 mL/min; potential +1.33 V vs Ag/AgCl; sensitivity 5000 nA; mobile phase: 0.015M pentanesulfonic acid sodium salt, 0.2M Na_2SO_4, and 0.1% acetic acid.

greatly affect the peak height that was observed at the 0.3M concentrations.

Liquid Chromatography.—Peculiar peak shapes noted in the fluorescence detection of OPA-derivatized aminoglycosides were occasionally seen in the chromatogram from the LCED method described here and varied with mobile phase composition or instrumental parameters. To determine whether the anomalous peaks were instrument artifacts, the separate purified C fractions were injected. They exhibited the same characteristics under identical chromatographic conditions. However, double peaks for the C_2 constituent have been reported in chromatographic separations (11) which may represent the C_{2b} and C_{2a} fractions (7). A typical chromatogram of gentamicin is shown in Figure 2.

Response vs Concentration.—When the signal vs concentration relationship was studied over the range of 16–60 μg gentamicin injected, the relationship in the 30–60 μg value became nonlinear. Anhalt and Brown (17) noted that erroneous assays of aminoglycosides resulted from their tendency to adsorb on the surfaces of glass vessels. By using peak areas and a linear regression analysis, we determined that the usable analytical concentration range was limited to 16–30 μg gentamicin sulfate injected. Correlation coefficients (r) of 0.999 were obtained for the gentamicin components; however, the linear regression did not project through the origin. Below 16 μg of the antibiotic injected, the signal-to-background noise ratio became unfavorable.

Evaluating Standard and Samples.—Because gentamicin sulfate is a multi-component antibiotic, the fractional composition of the USP Reference Standard had to be evaluated by LCED. Data already exist for the analysis of the standard by the official method. To determine the composition of the standard by HPLC, a calibration curve was prepared of the peak areas vs concentration for the individual C_{1a}, C_2, and C_1 fractions over the range 16–30 μg. The peak areas of the C_{1a}, C_2, and C_1 fractions in the standard were correlated to the calibration curve of its respective "C" fraction to ascertain the amount of each major component in the standard. These values were totaled and the percent by weight of each fraction was determined by comparison of its contribution to this total. The results are shown in Table 1. Other peaks appearing in the standard chromatograms possibly relate to the C_{2a} and C_{2b} fractions but no positive identification was made.

A set of 5 samples was assayed on 2 different days with the gentamicin standard as the reference material. A linear calibration for the standard was prepared each day and the sample peak areas of the "C" fractions were compared to those

Table 2. Determination of gentamicin sulfate samples by LCED method

Day	Sample	Gentamicin, sulfate, mg/mL	Gentamicin components, % by wt		
			C_{1a}	C_2	C_1
1	A	2.99	23.4	35.5	41.1
	B	2.66	25.9	41.3	32.7
	C	3.31	24.7	37.4	37.8
	D	2.81	26.7	34.7	38.5
	E	2.57	26.6	39.9	33.5
2	A	3.09	22.9	35.2	41.8
	B	2.70	25.2	42.2	32.6
	C	2.76	24.5	38.5	37.0
	D	2.78	26.2	34.6	39.2
	E	2.96	25.4	40.3	34.3

Table 3. Paper chromatographic separation and microbiological assay of gentamicin sulfate samples [a]

Sample	Gentamicin components, % by wt		
	C_{1a}	C_2	C_1
A	29.04	33.41	37.55
C	29.20	36.00	34.80
E	30.51	38.52	30.96

[a] Mobile solvent phase for paper chromatography consisted of 200 mL chloroform, 100 mL methanol, and 100 mL 17% aqueous ammonia.

of the standard to calculate the amount of each present. The calculated weights of C_1, C_2, and C_{1a} in the samples were totaled and the individual percentages of the components determined. The results obtained on the 2 test days are shown in Table 2. The reproducibility of the sample analyses was good. The corresponding microbiological assay results after paper chromatography are given in Table 3 for Samples A, C, and E. The failure of the absolute values to agree is to be expected, because the paper chromatography as performed in the FDA official method (5) does not separate or distinguish the minor constituents present, such as C_{2a} and C_{2b}. It is significant, however, that the relative percentages of the fractions of Samples A, C, and E as determined by both methods compare favorably. For example, Sample E has the highest percentages of C_{1a} and C_2 and the lowest percentage of C_1 as determined by both methods. In addition, Sample A has the highest percentage of C_1 and the lowest percentage of C_2 as determined by these procedures.

In conclusion, the reverse phase LCED procedure presented here offers a rapid and precise method for the assay of the C_{1a}, C_2, and C_1 fractions of gentamicin sulfate. No derivatization technique is necessary for this analysis and a single pump system may be used. Results obtained are reproducible and the linear response is comparable to the fluorescence detection assay.

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DRUG RESIDUES IN ANIMAL TISSUES

Determination of Penicillin G, Ampicillin, and Cephapirin Residues in Tissues

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A cylinder plate microbiological method was developed for the rapid, quantitative determination of penicillin G, ampicillin, and cephapirin in animal tissues. The method uses agar plates seeded with stable spores of *Bacillus stearothermophilus* var. *calidolactis* and incubated 4 h at 64°C. Standard curves were obtained for the following ranges of concentration of antibiotics in tissues: 0.02-0.32 IU penicillin G/g, 0.0125-0.2 μg ampicillin/g, and 0.02-0.32 μg cephapirin/g. The proposed method is suitable not only for penicillin residue analysis, for which the sensitivity has been greatly improved compared with the *Sarcina lutea* method, but also for depletion studies on these antibiotics, which are commonly used to treat diseases in food-producing animals.

The presence of drug residues in tissues of food-producing animals is highly undesirable from the standpoint of public safety. To protect consumers from unacceptable exposure to drug residues in ingested food, reliable analytical methods must be developed for detecting these residues. These methods must be rapid and inexpensive, must be capable of assaying large numbers of samples, and must have adequate sensitivity.

In Canada, the most commonly used methods for determining antibiotic residues are those described in a Food and Drug Administration publication, revised in 1968 (1).

Specification of *Bacillus stearothermophilus* var. *calidolactis* as the test organism has resulted in 2 major improvements in the screening procedures, namely, greater sensitivity and shorter incubation. A quantitative cylinder plate procedure for the determination of penicillin G, ampicillin, and cloxacillin residues in milk was developed in our laboratories (2). The method is well suited for both residues and milk-out studies.

In this paper, an improved procedure is described for determining trace levels of penicillin G, ampicillin, and cephapirin in tissues of food-producing animals. Sensitivity is improved, yet an extraction followed by a concentration step is not needed, as would have been required by the modified *Sarcina lutea* procedure to achieve the same sensitivity.

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Experimental

Reagents and Apparatus

(a) *Maintenance medium.*—Trypticase soy agar (BBL, division of Becton, Dickinson and Co. Canada, Ltd, 2464 S Sheridan Way, Mississauga, Ontario, Canada L5J 2M8).

(b) *Broth medium.*—Trypticase soy broth (BBL).

(c) *Assay medium.*—Seed agar, antibiotic medium A; formerly listed as Antibiotic Medium No. 1 (BBL).

(d) *Buffer solution.*—1% phosphate buffer, pH 6.0 (±0.1).

(e) *Antibiotic standards.*—Penicillin G potassium, ampicillin trihydrate, cephapirin sodium, and sodium cloxacillin, USP Reference Standards (U.S. Pharmacopeial Convention, Rockville, MD 20852).

Prepare stock solution of 1000 IU or μg/mL in 1% phosphate buffer, pH 6.0 (±0.1). Dilute stock solution to 10 IU or μg/mL solution in the 1% phosphate buffer. Further dilute the 10 IU or μg/mL solution in 1% phosphate buffer to obtain concentrations of 50 times the final concentrations.

(f) *Cylinder dispenser.*—Shaw-Dispenser (E.C. Condit, Middle Haddam, CT 06456).

(g) *Cylinders.*—Penicylinders, stainless steel, 8.0 mm od × 10 mm (Fisher Scientific Co. Ltd, 2660 Southvale Cr, Ottawa, Ontario, Canada K1B 4W5).

(h) *Zone reader.*—Antibiotic Zone Reader II (Fisher-Lilly).

(i) *Centrifuge.*—Model RC2-B with HS-4 rotor (Sorvall).

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(j) *Incubator.*—Model 3956 modified, capable of operating at 64°C (Forma Scientific, Marrietta, OH 45750).

(k) *Homogenizer.*—Sorvall Omni-mixer with 400 mL capacity stainless steel chambers (Sorvall).

(l) *Cornwall syringe.*—10 mL (Fisher Scientific Co. Ltd).

(m) *Petri dishes.*—Stacking dishes, No. 1054 (Falcon Plastic Co., 1950 Williams Dr, Oxnard, CA 93030), with porcelain tops (Canadian Laboratory Supplies, 2710 Lancaster Rd, Ottawa, Ontario, Canada K1B 4T7).

Preparation of Bacterial Spore Suspension

Inoculate 6 Erlenmeyer flasks, each containing 150 mL trypticase soy broth, with *Bacillus stearothermophilus* var. *calidolactis* ATCC 12980. Incubate at 64°C. Determine extent of sporulation by spore stains until ca 80% level is reached (72 h). Centrifuge 10 min at 5000 rpm. Decant supernate, wash cells twice with sterile normal saline, resuspend cells in 70 mL normal saline, and store at 4°C. Suspension is stable 4 weeks.

Preparation of Plates

Inoculate antibiotic medium A, cooled to 60°C, with 1.5 mL spore suspension of *B. stearothermophilus* var. *calidolactis*/100 mL medium. Pour 8 mL inoculated melted agar into each Petri dish, using Cornwall syringe, and let agar layer harden on level surface. Refrigerate plates 15 min before use.

Assay Procedure

Standard curve: Follow general instruction described for standard curves (1). After homogenization, heat mixture 5 min at 84°C, cool, and then centrifuge 10 min at 2000 rpm. Fill 3 alternate cylinders with standard and the other 3 cylinders with reference concentration. Incubate filled cylinders 4 h at 64°C. Calculate best straight line, using least squares linear regression analysis.

Sample: Homogenize 10 g tissue with 40 mL buffer (1:5). Heat 5 min at 84°C, and centrifuge. Proceed as for the standard, filling 3 alternate cylinders with sample and other 3 cylinders with reference concentration. Use 3 plates for each sample. Correct average value in relation to reference value of standard. Calculate corresponding concentration. Multiply by dilution factor (5) to obtain final concentration.

Results and Discussion

The standard curves were constructed by adding known amounts of the respective anti-

Table 1. Estimated concentrations for beef tissues spiked with penicillin G

Theor. concn, IU/mL	Mean adjusted zone size, mm	Estd concn, IU/mL[a]	CV, %
	Muscle		
0.004	12.6	0.0041	4.45
0.008	14.8	0.0079	7.70
0.016	17.3	0.0162	2.33
0.032	19.7	0.0330	7.36
0.064	21.8	0.0630	7.51
	Kidney		
0.004	12.1	0.0039	10.11
0.008	14.6	0.0082	11.24
0.016	17.1	0.0163	6.00
0.032	19.4	0.0323	6.02
0.064	21.8	0.0629	8.36
	Liver		
0.004	11.9	0.0039	20.31
0.008	14.4	0.0080	21.63
0.016	16.6	0.0157	14.60
0.032	19.2	0.0332	17.93
0.064	21.3	0.0617	16.44

[a] Average of 12 determinations.

Table 2. Estimated concentrations for beef tissues spiked with ampicillin

Theor. concn, μg/mL	Mean adjusted zone size, mm	Estd concn, μg/mL[a]	CV, %
	Muscle		
0.0025	12.4	0.0024	5.03
0.005	15.1	0.0053	6.75
0.01	17.1	0.0099	4.01
0.02	19.4	0.0192	6.12
0.04	21.9	0.0405	11.34
	Kidney		
0.0025	12.4	0.0024	2.95
0.005	15.2	0.0055	3.61
0.01	17.2	0.0097	1.55
0.02	19.4	0.0187	7.47
0.04	22.2	0.0416	1.98
	Liver		
0.0025	12.2	0.0025	9.79
0.005	14.9	0.0052	5.03
0.01	17.2	0.0098	1.07
0.02	19.7	0.0200	6.18
0.04	22.1	0.0391	4.24

[a] Average of 9 determinations.

Table 3. Estimated concentrations for beef tissues spiked with cephapirin

Theor. concn, μg/mL	Mean adjusted zone size, mm	Estd concn, μg/mL[a]	CV, %
	Muscle		
0.004	13.0	0.0041	8.37
0.008	15.2	0.0081	8.23
0.016	17.1	0.0149	4.67
0.032	19.4	0.0315	5.19
0.064	21.7	0.0668	8.75
	Kidney		
0.004	12.8	0.0040	7.10
0.008	15.1	0.0082	4.82
0.016	17.2	0.0152	1.59
0.032	19.7	0.0319	4.73
0.064	22.0	0.0656	3.85
	Liver		
0.004	11.9	0.0038	9.30
0.008	14.8	0.0085	4.59
0.016	17.1	0.0161	0.59
0.032	19.6	0.0326	3.24
0.064	22.0	0.0633	2.69

[a] Average of 9 determinations.

biotic to antibiotic-free animal tissues. The tissues, obtained from local supermarkets, were carefully tested for the presence of inhibitory substances before being used in this study. Tissue controls were run with each experiment. The resulting zone sizes were processed mathematically using least squares linear regression of zone size vs log concentration. This procedure resulted in correlation coefficients ranging from 0.9991 to 0.9999 (0.9982 for ampicillin in beef kidney) for penicillin G in beef and pork muscle, kidney, and liver and in chicken muscle and liver; for ampicillin in beef muscle, kidney, and liver; and for cephapirin in beef muscle, kidney, and liver.

Table 5. Comparison of *B. stearothermophilus* var. *calidolactis* method with *S. lutea* method, using penicillin G in beef muscle

	Concn, IU/mL		
Method	0.064	0.016	0.004
S. lutea	0.0625	0.0168	neg.
B. stear.	0.0633	0.0163	0.0041

A total of 9 determinations of 5-point standard curves for each antibiotic was obtained using 3 different batches of bacterial spore suspension as the inoculum. The precision and accuracy of the method for each individual antibiotic in 3 bovine tissues are illustrated in Tables 1, 2, and 3. Results are expressed in IU/mL or μg/mL. To convert these measurements to the final concentration values, multiplication by 5, the dilution factor, is required. The coefficients of variation (CV) are generally lower than 11%, with the exception of penicillin G in liver. However, these higher variations still fulfill the requirements for intralaboratory reproducibility of analytical results by being less than 20–25%. These requirements were recently reviewed by Horwitz et al. (3).

Because the reproducibility data were calculated from standard curves prepared by averaging 9–12 determinations with different bacterial spore suspensions and on different days, coefficients of variation can be lowered by using standard curves established with the same bacterial spore suspension on the same day. Thus,

Table 4. Recovery, minimum detectable level (MDL), and working range for penicillin G, ampicillin, cephapirin, and cloxacillin in beef tissues, *B. stearothermophilus* var. *calidolactis* cylinder plate method

Antibiotic	Tissue	Rec., %	MDL, μg/mL	Working range μg/mL	Working range μg/g
Penicillin G	muscle	98.0	0.0014	0.004–0.064[a]	0.02–0.32[b]
	kidney	96.4	0.0016	0.004–0.064[a]	0.02–0.32[b]
	liver	94.6	0.0017	0.004–0.064[a]	0.02–0.32[b]
Ampicillin	muscle	96.5	0.0009	0.0025–0.04	0.0125–0.2
	kidney	96.9	0.0009	0.0025–0.04	0.0125–0.2
	liver	96.4	0.0010	0.0025–0.04	0.0125–0.2
Cephapirin	muscle	98.9	0.0011	0.004–0.064	0.02–0.32
	kidney	99.1	0.0013	0.004–0.064	0.02–0.32
	liver	97.1	0.0017	0.004–0.064	0.02–0.32
Cloxacillin	muscle	96.6	0.008	0.03–0.48	0.15–2.4

[a] IU/mL.
[b] IU/g.

Table 6. Study of interference of sulfamethazine, sulfathiazole, nitrofurazone, and furazolidone added at various levels with penicillin G to beef tissues (*B. stearothermophilis* var. *calidolactis* method)

	Penicillin G, 0.016 IU/mL, and										
	Sulfamethazine			Sulfathiazole			Nitrofurazone		Furazolidone		
Tissue	100[a]	50	10	100	50	10	50	10	50	10	Penicillin G, 0.016 IU/mL
Muscle	16.7[b]	16.7	16.8	16.7	16.6	16.7	17.1	16.8	19.0	16.9	16.7
Liver	16.6	16.8	16.7	16.8	16.7	16.7	16.7	16.7	18.3	16.7	16.6
Kidney	16.8	16.9	16.9	17.0	16.9	16.9	17.0	17.0	19.6	17.1	16.9

[a] Values are μg/mL.
[b] Values are zone size (mm).

in the case of penicillin G in liver, the following CV values for the 5 points of the standard curve are obtained: 5.00, 6.17, 7.00, 5.06, and 5.65%. It is therefore important to check the performance of the method for each particular application to determine the frequency of re-establishing the standard curve. New standard curves should always be established with each new batch of bacterial spore suspension and used for calculating results.

The minimum detectable levels (MDL) are listed in Table 4. These values were calculated by an extrapolation technique, resulting in concentrations corresponding to a 9.0 mm zone which we consider to be the lowest size capable of being reliably measured by the zone reader. The practical working ranges are also indicated in Table 4, and they correspond to concentrations used in the preparation of the standard curves. The recovery values were obtained by comparison of the concentration estimates in buffer with those in respective tissues.

A comparison study was carried out with penicillin G in muscle tissue at 3 known concentration levels, using *B. stearothermophilus* and *S. lutea* methods (Table 5). Excellent agreement was obtained at the 2 higher concentrations, although the *S. lutea* method failed to detect a concentration of 0.004 IU/mL. All concentrations measured by both methods were very close to theoretical values.

To test for possible interference by other antimicrobial drugs commonly used in veterinary practice, we carried out 4 studies in 3 tissues (Table 6). No positive interference by sulfamethazine, sulfathiazole, or nitrofurazone was detected, even in the 50–100 ppm range. Furazolidone showed slight positive interference at 50 ppm, but none at 10 ppm.

During the preliminary experiments with *B. stearothermophilus*, a nonspecific inhibition effect was observed with beef kidneys. This effect was eliminated by incorporating a heating step into the procedure. A similar effect was observed during the summer months when the *S. lutea* method was used. It prevented the establishment of the limit of detectability for this method at the level specified by the FDA Manual (1). The comparative study with the *B. stearothermophilus* method had to be postponed to later months, when the nonspecific inhibition effect on the *S. lutea* method was no longer present.

The proposed method is suitable for both residue analysis and depletion studies. Sensitivity has been greatly improved when compared with the *S. lutea* method, and the method is also more rapid; results are available within 6 h. Chances of interference from microbiological contamination are minimized by the relatively high incubation temperature and the short incubation time.

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COSMETICS

Gas-Liquid Chromatographic Method for Determining 1,4-Dioxane in Cosmetics

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A gas-liquid chromatographic procedure has been developed for the quantitation of 1,4-dioxane in various cosmetic products including lotions, cleansers, skin creams, make-ups, and shampoos. The impurity is extracted into an aqueous phase followed by column cleanup to remove nonpolar interferents. 1,4-Dioxane is partitioned into toluene, passed through an extraction tube to remove water and other polar compounds including organic dyes, concentrated by adsorption onto silica, further purified by washing with dichloromethane, and eluted with acetonitrile for injection into the gas chromatograph. The mean recovery of 1,4-dioxane from 51 cosmetic products, determined by spiking, was 63%. The limit of detectability is about 0.5 ppm and the minimum quantifiable level is about 2 ppm. The identity of 1,4-dioxane is confirmed by mass spectrometry.

It has been estimated that about one-third of emulsion-based cosmetics contain polyoxyethylene derivatives that may be contaminated by 1,4-dioxane (1). Depending on the manufacturing process used, 1,4-dioxane may be formed during the polymerization of ethylene oxide to produce the polyoxyethylene portion of the emulsifier molecules (2). 1,4-Dioxane is a carcinogen in rats and mice, producing squamous-cell carcinomas of the nasal turbinates in the former and hepatocellular carcinomas in the latter (3, 4).

Several gas chromatographic methods have been reported for the determination of 1,4-dioxane in ethoxylated surfactants. These are based on vacuum distillation (2), direct injection of the surfactant with precolumn treatment to separate the 1,4-dioxane from the surfactant matrix (5, 6), or injection after dilution, with detection by mass spectrometry (7). Of 70 ethoxylated cosmetic raw materials analyzed by the U.S. Food and Drug Administration, 44% contained more than 10 ppm 1,4-dioxane (8). Of direct concern to the consumer, however, are the levels of 1,4-dioxane in commercially available cosmetic products. A method designed for the determination of 1,4-dioxane in cosmetics is described in this paper. The method provides for substantial sample cleanup before injection to avoid possible interfering substances which may be present in complex cosmetic matrices. GLC–mass spectroscopy is used to confirm the identity of 1,4-dioxane, as required.

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METHOD

Apparatus

(a) *Gas chromatograph.*—Microprocessor-controlled Hewlett-Packard Model 5880A equipped with printer-plotter mechanism and flame ionization detector (Hewlett-Packard, Palo Alto, CA). Coiled glass column (0.91 m × 4 mm id), packed with Chromosorb 106 porous polymer (Chromatographic Specialties, Brockville, Ontario, Canada) and adapted to the chromatograph with short length of nickel tubing. Column, injection port, and detector temperatures 210°C; nitrogen, 60 mL/min; hydrogen, 35 mL/min; and air, 400 mL/min. Columns were conditioned 72 h at 210°C with nitrogen flow of 60 mL/min.

(b) *Centrifuge.*—IEC Model K (International Equipment Co., Needham Heights, MA).

(c) *Water bath.*—Buchler Co., Fort Lee, NJ.

Reagents

(a) *1,4-Dioxane.*—Scintanalyzed (Fisher Scientific Co. Ltd, Ottawa, Ontario, Canada).

(b) *Acetonitrile.*—HPLC grade (J.T. Baker Chemical Co., Phillipsburg, NJ). Use material free of propionitrile to avoid interference with internal standard peak.

(c) *Dichloromethane.*—Distilled in glass (Burdick & Jackson Inc., Muskegon, MI).

(d) *Ethyl acetate.*—Distilled in glass (Caledon-Laboratories Ltd, Georgetown, Ontario, Canada).

(e) *Toluene.*—Distilled in glass (Burdick & Jackson, Inc.).

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(f) *Boric acid.*—Analytical reagent (Fisher Scientific Co. Ltd).

(g) *Magnesium sulfate.*—Anhydrous laboratory reagent (Fisher Scientific Co. Ltd).

(h) *Silica gel.*—Silicar CC-7 (Mallinckrodt Chemical Works, St. Louis, MO).

(i) *Disposable extraction columns.*—20 mL Clin-Elut. No. 1020 (Analytichem International, Harbor City, CA).

(j) *Silica cartridges.*—Sep-Pak (Waters Scientific Ltd, Mississauga, Ontario, Canada).

(k) *Octadecyl silane cartridges.*—Sep-Pak C_{18} (Waters Scientific Ltd).

(l) *Internal standard solution.*—Ethyl acetate in acetonitrile (10% v/v) prepared by pipetting 10 mL ethyl acetate into 100 mL volumetric flask and diluting to volume with acetonitrile.

(m) *Magnesium sulfate and boric acid solution.*—Dissolve 13 g magnesium sulfate and 3.3 g boric acid in water, mix, and dilute quantitatively to 100 mL.

(n) *Spiking solution.*—1,4-Dioxane in water (0.02% v/v) prepared by quantitative dilution of 1,4-dioxane. This corresponds to a level of 13.7 ppm 1,4-dioxane in 15 g cosmetic (taking density of dioxane as 1.028 g/mL).

(o) *Calibration solutions.*—Prepare, by quantitative dilution, solutions containing 0.00125 and 0.0025% (v/v) of 1,4-dioxane in acetonitrile and add 2.5 μL internal standard solution for each mL calibration solutions. These solutions correspond to 6.9 and 13.7 ppm 1,4-dioxane in 15 g cosmetic, assuming 100% recovery.

Linearity

Prepare solutions containing 0.0025, 0.00625, 0.0125, 0.0375, and 0.0625% (v/v) 1,4-dioxane in acetonitrile by quantitative dilution and add 2.5 μL internal standard solution for each mL of these solutions. These solutions correspond to 13.7, 34.3, 68.5, 205.6, and 342.5 ppm 1,4-dioxane in 15 g cosmetic. Inject 10 μL duplicates of each solution into chromatograph and compute response factors relative to internal standard. Relative standard deviation of mean response factor should not exceed 2%.

Procedure

To prepurify glass-distilled toluene, pass 1200 mL through 2.5 cm diameter column containing 50 g Silicar CC-7 silica gel. Accurately weigh ca 15 g cosmetic into 50 mL screw-cap tube (28 × 120 mm). Pipet 15 mL magnesium sulfate–boric acid solution into tube and shake vigorously by hand for 3 min. Heat in water bath at 70°C for 10 min, shake vigorously for 1 min, and centrifuge for 5 min at 3000 rpm. By means of 30 mL syringe fitted with 6 in., 20 gauge needle, transfer lower (aqueous) phase through C_{18} Sep-Pak cartridge (preconditioned by passage of 20 mL distilled water) into 250 mL centrifuge vessel. Rinse syringe and cartridge with 2 mL water and add rinse to centrifuge vessel. Add 60 mL prepurified toluene to centrifuge vessel, shake vigorously by hand for 3 min, centrifuge for 5 min at 3000 rpm, pass toluene (upper) layer through No. 1020 Clin-Elut tube, and collect. Repeat extraction of aqueous layer with additional 60 mL toluene, pass through Clin-Elut tube, and add to first extract. Add sufficient toluene to Clin-Elut tube to collect total volume of 140 mL toluene. Using 50 mL syringe with 3 silica Sep-Pak cartridges attached in series, pass entire toluene extract through to discard. Rinse syringe and cartridges with two 5 mL portions of dichloromethane. Elute 1,4-dioxane from cartridges with 8 mL acetonitrile, collect in 20 mL screw-cap test tube (150 × 15 mm), and mix with 20 μL internal standard solution. Inject 10 μL of each calibration solution and sample preparation into chromatograph and compute dioxane level in the cosmetic in ppm.

Recovery Efficiency Determination

Spike a second accurately weighed 15 g portion of cosmetic with 1 mL spiking solution and repeat analysis. If the cosmetic contains ≥30 ppm 1,4-dioxane, spike with higher level of 1,4-dioxane, keeping concentration of internal standard unchanged and volume of spiking solution added at 1 mL. Calculate recovery efficiency in percent from 100 $(C_T - C_I)/C_S$, where C_T, C_I and C_S are the total, initial, and spiked concentrations, respectively, of 1,4-dioxane in ppm.

Results and Discussion

Cosmetic products were shaken with magnesium sulfate to separate them into their constituent phases. Typically, these consisted of a lower phase and an upper oil phase, with a band of particulate matter at the interface. Boric acid, added with the magnesium sulfate, forms a water-soluble ionic complex with ethylene and propylene glycol (9), present in some cosmetics. These compounds, if not complexed, partition into toluene and may interfere with the 1,4-dioxane peak in the chromatogram. The entire aqueous layer, rather than an aliquot of it, was transferred to the extraction column because the amount of water in cosmetic products varies from one to the next. In most cases, phase separation

was good and the loss of 1,4-dioxane associated with this transfer is probably less than 5% of the total. In rare cases when only an aqueous phase was present, the entire sample was transferred to the extraction column. The aqueous phase was passed through a C_{18} Sep-Pak cartridge which retains nonpolar compounds but little of the 1,4-dioxane. In the range corresponding to 13.7–68.5 ppm 1,4-dioxane in the original cosmetic sample taken, 2–4% of the 1,4-dioxane is retained on the cartridge. The amount of 1,4-dioxane removed from the aqueous phase after 2 extractions with 60 mL toluene depends on the amount of water in the cosmetic. The coefficient for the partition of 1,4-dioxane between water and toluene at room temperature was 1.33, as determined experimentally by shaking 15.42 mg 1,4-dioxane between 15 mL water and 100 mL toluene and analyzing the 2 phases. On this basis, recoveries of 90 and 86% would be anticipated from products containing 40 and 80% water, respectively. The presence of magnesium sulfate and boric acid, as well as water-soluble materials in the cosmetic would result in unique partition behavior for each cosmetic.

The toluene solution is passed through the Clin-Elut column to remove the water present after the aqueous extraction. This is necessary to prevent an attack by residual water on the silica cartridges. The Clin-Elut column also removes organic colored materials and other polar compounds. 1,4-Dioxane was not retained on the column. This was demonstrated by passing a solution of toluene containing 5.14 mg 1,4-dioxane, corresponding to 343 ppm 1,4-dioxane in a cosmetic sample, through the column and then eluting with a further 50 mL toluene. Upon completion of the analysis, no 1,4-dioxane was detected in the second 50 mL eluate. The Sep-Pak cartridges are used to remove 1,4-dioxane from the toluene. Three are needed because, with the large volume (140 mL) of toluene involved, the 1,4-dioxane chromatographs through the cartridges. The cartridges also separate the 1,4-dioxane from nonpolar compounds which remain in the toluene. The amount of 1,4-dioxane not adsorbed was estimated for the case of samples originally containing 5.14 mg 1,4-dioxane by passing the toluene effluent through a second series of 3 cartridges and completing the analysis in the usual way. An additional 0.4% of the original 1,4-dioxane was recovered. The cartridges are washed with dichloromethane to remove residual toluene. This is necessary because the toluene contains an impurity, thought to be benzene on the basis of its retention time, which interferes with the 1,4-dioxane peak on the chromatogram. At 1,4-dioxane loadings corresponding to 343 ppm in a cosmetic sample, about 7% of the adsorbed 1,4-dioxane was lost to the dichloromethane as estimated by direct injection of the dichloromethane eluate. However, this estimate is only approximate, due to interference with the 1,4-dioxane peak by eluted impurities. Removal of adsorbed 1,4-dioxane from the cartridges by acetonitrile was complete. Subsequent 5 mL portions of acetonitrile eluted and injected into the gas chromatograph contained no detectable 1,4-dioxane.

The chromatographic response in the range equivalent to 2.055–13.7 ppm 1,4-dioxane in 15 g cosmetic (0.038–0.25 μg on column) was linear with a slight positive intercept, possibly due to an integrator artifact (ppm = 139.4 area ratio [1,4-dioxane/internal standard] + 0.363). Two or more calibration points were determined daily and appropriate linearity parameters were calculated. Five injections of a sample containing the equivalent of 2.055 ppm 1,4-dioxane gave an RSD of 1.6%. Typical chromatograms are presented in Figure 1.

The reproducibility and bias of the method were determined by analyzing known solutions of 0.2056 mg 1,4-dioxane in 7 mL water. These solutions, corresponding to 13.7 ppm 1,4-dioxane in 15 g cosmetic, were analyzed 6 times at intervals over a period of several weeks. The mean recovery was 85.1% with an RSD of 2.4%. Recoveries at 1,4-dioxane levels corresponding to 68.5 ppm were 82.5% with an RSD of 2.7% for 5 assays.

Cosmetic products contain individual amounts of water and other ingredients that influence the partition and extraction of 1,4-dioxane. It is therefore necessary to determine the recovery of 1,4-dioxane from each product analyzed by spiking. A known amount of 1,4-dioxane in 1 mL water was shaken into the cosmetic before analysis. The mean recovery from 51 cosmetic samples including lotions, cleansers, skin creams, make-ups, and shampoos was 63.0% with an RSD of 19.9% (6 samples, <50%; 11 samples, 50–60%; 20 samples, 60–70%; 11 samples, 70–80%; 3 samples, >80%). A commercial skin-moisturizing product containing no detectable 1,4-dioxane was spiked at levels corresponding to 13.7, 68.9, and 342.7 ppm and analyzed 3 times at each level. The mean percent recoveries, with the RSD in parentheses, were 69.4 (4.0), 74.2 (2.9), and 70.2 (4.4), respectively.

The recovery of 1,4-dioxane from a few cosmetic products was less than 20%. In most of

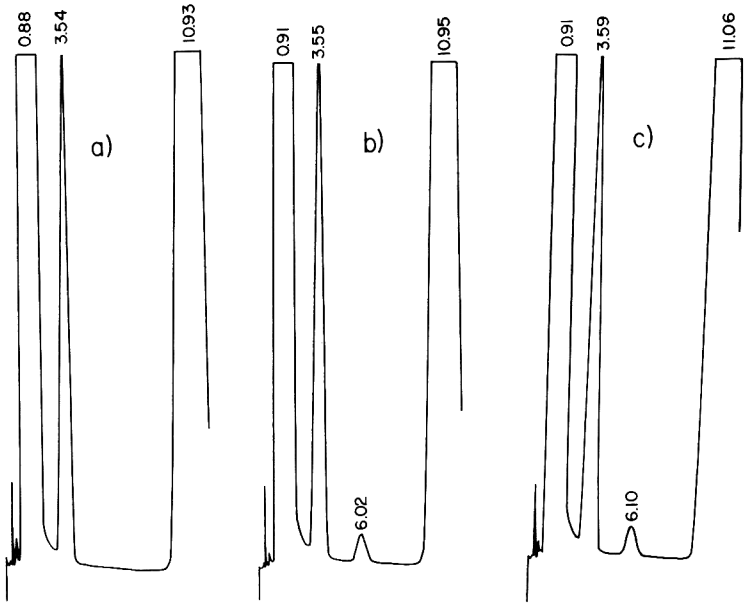

Figure 1. Typical chromatograms: (a), cosmetic product containing no 1,4-dioxane; and (b), the same product spiked with 13.7 ppm 1,4-dioxane. Peaks are: 0.9 min, acetonitrile and dichloromethane; 3.55 min, ethyl acetate internal standard; 6.02 min, 1,4-dioxane; and 10.95 min, toluene. Extraction efficiency 76.8%. (c), Water standard containing equivalent of 13.7 ppm 1,4-dioxane; recovery 86.0%.

these cases, injection of the toluene layer into the gas chromatograph after extraction of the aqueous phase revealed the presence of ethanol. Ethanol changes the polarity of the toluene sufficiently to prevent adsorption of 1,4-dioxane on the cartridges. The recovery of 1,4-dioxane decreases from about 90% of theoretical at 0 or 30 mg ethanol in 120 mL toluene to 73, 32, and 6% at ethanol levels of 60, 150, or 300 mg, respectively. Taking the room temperature water/toluene partition coefficient of ethanol to be 24, the 60, 150, and 300 mg levels would correspond to 2, 5, and 10% ethanol in the original cosmetic sample. About 83% of the ethanol can be removed from the toluene by extraction with 25 mL water. Removal of this amount of ethanol is usually sufficient to allow the analysis to proceed but there is a concomitant loss of about 22% of the 1,4-dioxane present. Thus, when it is necessary to incorporate a water extraction of the toluene into the method, the recovery of 1,4-dioxane will be a maximum of 50 to 60% of the amount originally present in the cosmetic product. The actual mean recovery from 5 products analyzed in this way was 52%.

Samples containing more than 10 ppm 1,4-dioxane were subjected to mass spectral analysis on a Hewlett-Packard GC-MS system 5985 at an ionization potential of 70 eV to confirm the identity of 1,4-dioxane. Final solutions of 1,4-dioxane in acetonitrile were chromatographed into the mass spectrometer through a 1.2 m × 2 mm id glass column packed with Chromosorb 106 porous polymer and operated isothermally at 210°C. The spectra were identical to that of a 1,4-dioxane standard.

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DRUGS IN FEEDS

Turbidimetric Assay of Penicillin in Feeds: Addition of Magnesium Sulfate to Eliminate Chlortetracycline Interference

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The official AOAC method for extracting penicillin from feeds allows some chlortetracycline to be co-extracted if the latter is also present in the sample. This can cause a high bias in results obtained by using the turbidimetric assay in which the test organism is sensitive to both antibiotics. In this report, we show that magnesium ions can be used to circumvent the interference of chlortetracycline in the turbidimetric assay for penicillin.

The official AOAC agar diffusion plate method for penicillin in feeds is labor-intense, time-consuming, and not easily adapted to automation (1). A faster method would be useful to laboratories where the number of samples analyzed justifies the expense of automation and to laboratories needing a more rapid manual method. Turbidimetric assays provide both quick sample turnaround and an opportunity for automation. AOAC has adopted turbidimetric methods for chlortetracycline (2) and monensin (3), but not for penicillin.

A turbidimetric assay for penicillin in feeds has been described in detail (4). *Staphylococcus aureus*, the organism used in the method, is sensitive to chlortetracycline (CTC) in addition to penicillin, while the organism *Sarcina lutea*, which is used in the AOAC plate method, is not sensitive to CTC. However, *S. lutea* does not demonstrate the growth characteristics desired in a turbidimetric assay. Unfortunately, most of the feed samples analyzed by our laboratory contain both CTC and penicillin in a 2:1 ratio (g/ton).

Initial attempts using the turbidimetric assay for penicillin indicated that results were consistently greater for feeds guaranteed to contain CTC, and preliminary efforts to eliminate potential CTC interferences through the isolation of a *S. aureus* mutant resistant to CTC, but still sensitive to penicillin, failed. Therefore, an alternative approach for decreasing the response of *S. aureus* to CTC was investigated.

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Magnesium ions can inactivate the antibacterial activity of CTC in a turbidimetric assay (5). In this paper, we demonstrate that CTC is present in feed sample extracts used for the determination of penicillin, and that co-extracted CTC can cause an overestimation of the amount of penicillin present. Furthermore, we report on the use of added magnesium ions to circumvent the interference of CTC in the turbidimetric assay for penicillin.

METHOD

Apparatus and Reagents

(a) *Automatic turbidimetric system.*—AUTOTURB® System (Elanco Products Co., Div. of Eli Lilly and Co., 7405 Alabama St, Indianapolis, IN 46206). System pipets samples into assay tubes, dilutes with inoculated broth (66- and 100-fold dilutions using 0.15 and 0.1 mL sample loops, respectively), incubates tubes, and measures turbidity (6, 7). Include set of standard solutions for each assay tube carrier. After dilutor unit has processed samples, place tubes in the system water bath and incubate at 37.5°C until turbidity reaches 40–50% *T* for zero tubes. Stop growth by heating tubes 2 min in 80°C water bath. Cool tubes rapidly in water to room temperature and immediately read % *T* on the Autoturb reader with wavelength set at 600 nm.

(b) *Penicillin standard solution.*—Stock standard solution: penicillin standard (No. Pen-P, 500 μg/mL or 504.5 units procaine salt, Sigma Chemical Co., St. Louis, MO 63178)/mL. Working standard solutions: Dilute stock standard solution with pH 6 dilution buffer to concentrations of 0.2, 0.4, 0.6, and 0.8 μg/mL.

(c) *Penicillinase solution.*—5×10^5 enzyme units (No. 0346-63, Difco Laboratories, Detroit, MI)/mL. Add 0.1 mL of this concentrated stock solution directly to diluted feed extracts to give 5×10^3 units/mL final concentration where indicated.

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Table 1. Effect of penicillinase and magnesium ions on penicillin activity in a feed sample containing both penicillin and CTC

Sample	% Transmission	
	0.1 mL loop	0.15 mL loop
Diln buffer	41.6; 41.4	42.1; 42.4
Diln buffer + penase[a]	41.8; 40.8	40.5; 41.5
Pen. std soln[b]	51.5; 52.4	64.1; 63.8
Pen. stn soln + penase	41.4; 40.3	40.8; 40.6
Feed[c]	47.4; 46.8	51.2; 50.6
Feed + penase	44.6; 44.5	47.4; 47.2
Feed + $MgSO_4$ (250 mM)	44.4; 45.1	46.9; 46.8
Feed + penase + $MgSO_4$ (250 mM)	42.5; 42.3	43.7; 42.9

[a] Penase = penicillinase, 5×10^3 units/mL in sample tube.

[b] Penicillin standard solution, 0.8 μg/mL.

[c] Feed sample extracted and diluted according to AOAC method **42.280** to penicillin concentration between 0.2 and 0.8 μg/mL.

Table 2. Effect of penicillinase and magnesium ions on penicillin activity in a feed sample containing penicillin, but no CTC

Sample	% Transmission	
	0.1 mL loop	0.15 mL loop
Diln buffer	52.1; 51.2	52.1; 51.4
Feed	87.9; 87.6	93.5; 93.8
Feed + penase	52.5; 51.8	50.4; 51.3
Feed + penase + $MgSO_4$ (250 mM)	50.7; 50.5	53.0; 52.9

(**d**) *Dilution buffer.*—pH 6 phosphate buffer as described in **42.197** (8).

(**e**) *Growth medium.*—Antibiotic Medium 3; as in **42.196(n)** (8), in some cases medium was supplemented with 1.0 mM $MgSO_4$ as indicated in results.

(**f**) *Test organism.*—*Staphylococcus aureus* ATCC No. 9144 (American Type Culture Collection). Maintain as described elsewhere (9).

(**g**) *1M $MgSO_4$ solution.*—Dissolve 138 g $MgSO_4.H_2O$ in dilution buffer and dilute to 1 L in same buffer. Add this stock solution to samples to give specified final $MgSO_4$ concentration.

Preparation of Inoculum

Inoculate 10 mL growth medium with 1 loop *S. aureus* from slant culture. Incubate overnight at room temperature. Add 5–10 mL overnight culture to 1 L assay broth.

Preparation and Assay of Samples

Weigh feed samples, extract, and then dilute according to **42.280** (8). Mix $MgSO_4$ and/or penicillinase with feed extracts or standard solution at concentrations indicated before dilution in inoculated medium. For calculating potency of feed sample, 2 dose-response curves, one for 0.1 mL loop and the other for 0.15 mL loop, were obtained from percent transmission (% *T*) values corresponding to each working standard solution. Percent *T* values from duplicate tubes were averaged and used to prepare standard curves. A computer program was written to calculate the sample concentration (μg/mL) by interpolating from point to point of its respective standard curve. The potency of each sample was also calculated by computer using the dilution factor and the sample weight.

Results

Presence of CTC in Feed Extracts in Turbidimetric Assay for Penicillin

CTC may be co-extracted with penicillin when the AOAC method (1) is used for extracting penicillin from feed samples that contain both antibiotics. Results obtained using the turbidimetric procedure are invariably high compared with results obtained using the AOAC agar diffusion plate method.

Co-extraction of CTC was established by treating the extract of a feed sample (known to contain 50 g penicillin and 100 g CTC/ton) and a penicillin standard solution with penicillinase before assaying turbidimetrically for penicillin activity. When the penicillin standard solution was treated with penicillinase, no antibacterial activity could be detected (Table 1). As expected, the enzyme completely destroyed the growth-inhibition properties of penicillin. When the same concentration of penicillinase was added to the feed extract, antibacterial activity was reduced but not completely eliminated (Table 1). Furthermore, feed extracts containing only CTC showed no penicillin activity using the AOAC agar diffusion method, but inhibited growth of the assay organism in the turbidimetric method.

Extracting a feed known to contain penicillin but no CTC and treating the extract with penicillinase yielded results that indicated no antibacterial activity (Table **2**). The untreated extract gave a greater % *T* value, indicating significant growth inhibition. It appears that some of the CTC in the feed is co-extracted and interferes with the turbidimetric assay.

Table 3 Effect of $MgSO_4$ on penicillin activity

	% Transmission[a]			
	No penicillin		Penicillin (0.8 μg/mL)	
$MgSO_4$ (mM)[b]	0.1 mL loop	0.15 mL loop	0.1 mL loop	0.15 mL loop
0	34.1; 34.2	34.1; 34.2	54.9; 54.4	71.6; 71.9
150	31.9; 32.2	31.8; 31.2	54.9; 55.0	70.7; 71.4
200	30.0; 29.9	30.7; 31.4	55.3; 55.1	71.3; 71.3
250	30.2; 30.3	30.3; 30.1	55.7; 54.7	71.9; 72.2

[a] Growth medium was supplemented with 1 mM $MgSO_4$ (see text for explanation).

[b] Concentration in samples before dilution with inoculated media by automated diluter.

Effect of Magnesium Ions on Antibacterial Activity of Penicillin

One potential approach to correct for CTC contamination is to take advantage of magnesium ion inactivation of CTC activity in the turbidimetric method (5). Magnesium ions prevent binding of CTC to small ribosomal subunits (5). However, if magnesium is to be used to inactivate contaminating CTC in a feed extract used for penicillin assay, the following criteria must be considered and demonstrated experimentally: (a) Added magnesium ions do not increase or decrease penicillin activity. (b) Magnesium ions added to the inoculated growth medium as a result of its use to inactivate CTC in the feed extract do not otherwise affect bacterial growth.

It has been demonstrated that growth of the test organism is promoted by the addition of magnesium (in the form of $MgSO_4$) to the growth medium, and that growth promotion reaches a plateau when the $MgSO_4$ concentration reaches 0.4 mM (5). Therefore, in all experiments described in this report where extra magnesium ions were delivered to the growth medium due to the addition of magnesium to feed extracts (or standards) for CTC inactivation, the growth medium was supplemented with $MgSO_4$ at 1.0 mM to eliminate the growth-promotion effect. Results in Table 3 obtained using $MgSO_4$ supplemented growth medium show that addition of $MgSO_4$ to the penicillin standard does not change the post-incubation % T value over the range of $MgSO_4$ concentrations tested. Thus, the criteria on the use of magnesium to inhibit co-extracted CTC are met.

Use of Magnesium Ions to Eliminate CTC Effect in Penicillin Assay in Feed Extracts

If the residual antibacterial activity detected in a penicillinase-treated feed extract is due to co-extracted CTC (Table 1), one would expect that the addition of magnesium ions to the extract would inhibit the residual activity. On the other hand, if the residual activity is not due to co-extracted CTC, the addition of magnesium should not change the % T values. The results of such a test are shown in Table 1. When $MgSO_4$ was added to feed extract treated with enzyme, the % T values were essentially the same as for the buffer control (compare lines 1 and 8, Table 1). This is further evidence that co-extracted CTC is responsible for causing biased high results in the analysis of penicillin by using the turbidimetric method. Data in Table 1 also illustrate the effect of adding $MgSO_4$ to a non-penicillinase treated feed extract. The % T values change as expected for the presence of co-extracted CTC (compare lines 5 and 7, Table 1). Taken together, these experiments demonstrated the potential for eliminating co-extracted CTC activity by adding magnesium ions when determining penicillin by the turbidimetric method.

Table 4. Results (g/ton) for assay of penicillin in feed extracts with and without added magnesium

Sample[a]	Without $MgSO_4$	With $MgSO_4$[b]	Diff. (d)
1	57.5	47	10.5
2	49.5	38	11.5
3	80	80	0
4	46	55	−9
5	57.5	47	10.5
6	90.5	43.5	47
7	65	52.5	12.5
8	65	45	20
9	77.5	41.5	36
			$\bar{d}$ = 15.444
			S_D = 17.132
			t_8 = 2.550*

[a] All feeds labeled to contain both CTC and penicillin at 2:1 ratio.

[b] $MgSO_4$ concentration = 100 mM.

* Exceeds $t_{8,0.05}$ = 2.306; difference is significant at the 95% confidence level.

Nine feeds labeled to contain 50 and 100 g/ton of penicillin and CTC, respectively, were analyzed with and without the addition of magnesium ions (Table 4). Results for penicillin in 7 of the 9 feeds analyzed are lower with the addition of $MgSO_4$; the result for one feed is unchanged and for another, slightly increased (Samples 3 and 4, respectively). Table 4 also shows results of statistical analysis. Since t_8 exceeds $t_{8,0.05}$, the difference is significant at the 95% confidence level.

Discussion

S. aureus is the test organism of choice for the turbidimetric assay of penicillin in feeds because it forms a good suspension without aggregation, does not give a precipitate of extraneous material when the suspensions are heated at the end of incubation, and gives a nearly linear calibration curve in the range of concentrations used for the standard curve (10). All these properties are essential for a test organism in a turbidimetric assay. However, *S. aureus* is sensitive to CTC in addition to penicillin, and most of the feed samples contain both CTC and penicillin in a 2-to-1 ratio (g/ton).

In this paper we have shown that CTC is present in feed sample extracts used for the determination of penicillin. Several approaches could be used to overcome this interference problem: (*1*) Select another test organism that is sensitive only to penicillin and retains all those properties of *S. aureus* that are essential for a turbidimetric assay; (*2*) isolate a CTC-resistant mutant of *S. aureus*; (3) develop a different penicillin extraction procedure so that CTC will not be co-extracted; (4) inactivate the co-extracted CTC in the penicillin feed extract.

We have shown here that magnesium ions can be used to inactivate CTC without causing any adverse effects on the penicillin assay in the majority of the samples tested. We do not know why samples 3 and 4 in Table 4 result in equal and slightly higher assay values after treatment with magnesium ions. It is possible that other components in the feed may affect the amount of CTC co-extracted with penicillin, or the addition of magnesium ions may activate some growth-inhibiting factor in some feeds.

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FEEDS

Automated Determination of Crude Protein, Phosphorus, Calcium, Iron, and Magnesium in Feeds by Using Stopped-Flow Analyzer

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Methods are described for determination of crude protein, phosphorus, calcium, iron, and magnesium in feeds, using an automated microprocessor-based stopped-flow analyzer. Crude protein is determined by a reaction-rate procedure based on the ammonia-sodium phenate-hypochloride Berthelot reaction. Phosphorus determination is based on a phosphomolybdenum blue reaction-rate method. o-Cresolphthalein complexone, ferrozine, and calmagite are used as colorimetric reagents for calcium, iron, and magnesium, respectively. The methods are precise with relative standard deviations less than 1%, rapid with analysis rates of 110-266 samples per hour, sensitive, and require less than 1 mL sample and reagent volumes. Results for feed samples are comparable with those obtained by official AOAC methods.

The large number of animal feed samples presented for determination of several constituents in research, industrial, and regulatory laboratories has led to an increasing demand for rapid and precise analytical procedures. Various automated methods based on air-segmented continuous flow or flow-injection analysis have been developed in the routine analysis of agricultural products, and some of these have been adopted as official methods. In this paper, the use of a relatively simple stopped-flow analyzer is described for automated routine analyses of animal feeds. The stopped-flow technique has been shown in recent years to be a generally applicable analytical system, especially since microcomputer-controlled stopped-flow systems have been developed (1-5). The main advantages of the stopped-flow system are the rapid and thorough mixing of the reagent and sample, the ability to obtain measurement in a short time after mixing, the use of kinetic methods with all their advantages (6), and the high overall precision.

In this investigation, colorimetric methods for the determination of crude protein, phosphorus, calcium, iron, and magnesium in feeds have been adapted to an automated stopped-flow analyzer and evaluated in the analysis of AAFCO check feed samples. The importance of determining these analytes in feeds is based on the fact that they are essential nutrients needed for various cellular processes and perform functions in animal metabolism.

The determination of crude protein is based on a reaction-rate method developed for a stopped-flow system (7) and uses the ammonia-sodium phenate-hypochloride reaction. Phosphorus is determined using the phosphomolybdenum blue reaction in a reaction-rate procedure (8). For the determination of calcium, the *o*-cresolphthalein complexone reaction, introduced by Kessler and Wolfman (9) and improved by Gitelman (10), Moorehead and Biggs (11), and others, for the determination of calcium in serum is adapted to the stopped-flow analyzer.

The reaction of ferrous ions with ferrozine, a new sensitive reagent introduced by Stookey (12), is used for the determination of iron. This reagent is now widely used in the determination of serum iron (13-15). Magnesium determination is based on the magnesium-calmagite complex formation, a reaction introduced by Gindler and Heth (16) for the determination of magnesium in human blood serum. The method was introduced commercially by the Pierce Chemical Co. and has been adapted to a centrifugal analyzer (17).

METHODS

Principles

(1) *Crude protein.*—Samples are digested and the produced ammonia is determined by the Berthelot reaction in which ammonia reacts with hypochloride to form chloramine which, in turn, reacts with phenol under alkaline conditions to form indophenol. The stopped-flow analyzer aliquots and thoroughly mixes about 150 μL sample digest (premixed with phenol) with an

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equal volume of hypochloride and rapidly transfers the reaction product to the observation cell, where the reaction-rate measurement is performed on the indophenol product at 630 nm during 15 s after a 10-s delay.

(2) *Phosphorus.*—The method involves the reaction of phosphate with molybdenum to form 12-molybdophosphoric acid and a subsequent reduction by ascorbic acid, to form phosphomolybdenum blue. Sample digests are premixed with ascorbic acid and the stopped-flow unit is used to aliquot and mix equal volumes of molybdenum reagent and samples and transfer the reaction product to the observation cell. The absorbance change is measured at 650 nm for 4-s measurement time after 2-s delay time.

(3) *Calcium.*—Calcium is complexed with *o*-cresolphthalein complexone in the presence of 8-quinolinol to avoid interference of magnesium and cyanide by excluding heavy metals from the reaction. 2-Amino-2-methyl-1-propanol is used as buffering agent which maintains the pH at 10. Absorbance is measured at 580 nm during 1 s after a 5-s delay time.

(4) *Iron.*—Iron in the sample digests is reduced to ferrous form with ascorbic acid in an acid solution. The stopped-flow analyzer is used to mix equal volumes of pretreated samples with ferrozine reagent in a pH 4.5 acetate buffer. Absorbance is measured at 560 nm during 2.5 s after a 10-s delay time.

(5) *Magnesium.*—The method involves the reaction of magnesium with the mettalchrome dye calmagite which forms a pink complex with magnesium in alkaline medium. Ethyleneglycol-bis(*p*-aminoethylether) *N,N'*-tetraacetic acid (EGTA) virtually eliminates calcium interference, and cyanide complexes all heavy metals. The absorbance is measured at 520 nm during 2.5-s measurement time 5 s after the mixing of the solutions.

Apparatus

(a) *Stopped-flow analyzer.*—The instrument was designed in our laboratory (18). The entire system is automated using a Rockwell AIM 65 microcomputer (Rockwell International Co., Anaheim, CA 92803) for control of all operations, data acquisition and reduction, display, and printout of results. The sampling/mixing module of the analyzer is used to aliquot and mix ca 150 μL reagent and sample from the turntable, with a ratio accuracy better than 0.1%, and transfer the solution to an observation flow cell with a pathlength of 1.00 cm. Bandpass interference filters of about 10 nm half width are used in the photometer module to isolate the desired wavelength. Investigative equilibrium and reaction-rate programs are used to optimize each procedure, and interactive or dedicated routine programs are used for routine analysis.

In the routine reaction-rate program, the slope of the absorbance vs time, in the desired measurement time interval, is calculated by regression analysis using 30 points and is printed out as rate in milliabsorbance units per second (mA/s). In the routine equilibrium program, the absorbance measured is the average of a number of integrations in the desired time interval.

(b) *Dispenser/diluter.*—Model 25000 automatic pipet (Micromedic Systems Inc., Philadelphia, PA 19105), or equivalent.

(c) *Block digestor.*—Labconco, Kansas City, MO; Tecator, Inc., Boulder, CO; or equivalent.

(d) *Volumetric digestion tubes.*—75 or 250 mL (Model 1007-021, Tecator, Inc., or equivalent).

Reagents

Use deionized water for preparing solutions.

(1) *Crude protein.*

(a) *Phenol reagent.*—Mix 5 g phenol, 0.656 g NaOH, and 2 g sodium potassium tartrate in 100 mL water; prepare day before use. Sodium potassium tartrate is added to eliminate interferences to Berthelot reaction from side reactions.

(b) *Hypochlorite solution.*—2 g NaOH/100 mL commercial bleach containing sodium hypochlorite (e.g., Clorox, Clorox Co., Oakland, CA 94623). Prepare fresh daily.

(c) *Buffer solution.*—pH 11; 6.44 g sodium phosphate dibasic and 0.364 g NaOH/100 mL water.

(d) *Hypochloride-buffer reagent.*—Equal volumes of reagents **b** and **c**.

(e) *Potassium sulfate-mercuric oxide reagent mixture.*—95.5 + 4.5 w/w, respectively (e.g., Mixture No. 5, Pope Kjeldahl Mixtures, Inc., Dallas, TX 75221).

(f) *Nitrogen standard solutions.*—12.5–75.0 mg N/250 mL. Prepare 250 mg N/250 mL stock solution by adding 4.7172 g $(NH_4)_2SO_4$ primary standard (dried 2 h at 105°C before use) into 1 L volumetric flask. Add 37.68 g reagent e and 34.8 mL H_2SO_4 (so that standard is in same matrix as samples after digestion) and dilute with water. Prepare working standard solutions by appropriate dilutions with reagent **g**.

(g) *Blank digest solution.*—37.68 g reagent **e** + 34.8 mL H_2SO_4/L water.

(2) *Phosphorus.*—All standards and reagents must be stored in polyethylene bottles to prevent leaching of silicon from volumetric glassware.

(a) *Molybdenum reagent.*—1.209 g sodium molybdate dihydrate/100 mL 0.1M H_2SO_4. Prepare day before use to ensure equilibrium of different forms of molybdenum in solution.

(b) *Ascorbic acid reagent.*—Prepare daily with 0.53 g ascorbic acid, 0.656 g NaOH, and 5 g KI (to avoid precipitation of mercury in the diluted digest by complex formation) in 100 mL water.

(c) *Phosphorus standard solutions.*—20–100 ppm P. Prepare 100 ppm stock solution by adding 0.439 g potassium dihydrogen phosphate, primary standard (Baker Chemical Co., Phillipsburg, NJ 08865), dried 2 h at 105°C, in 1 L volumetric flask. Add 37.68 g potassium sulfate-mercuric oxide mixture (reagent *1*(e)) and 34.8 mL H_2SO_4 (so that standard is in same matrix as samples after digestion) and dilute with water. Prepare working standard solutions by appropriate dilution with reagent *1*(g).

(*3*) *Calcium.*

(a) *o-Cresolphthalein complexone reagent.*—Prepare stock solution by adding 0.0375 g *o*-cresolphthalein complexone (Sigma Chemical Co., St. Louis, MO 63178) to 30 mL water in 100 mL volumetric flask. Add 0.85 g 8-quinolinol and 2 mL HCl to 40 mL water, add to same volumetric flask, and dilute with water. Store in polyethylene bottle.

(b) *Aminomethylpropanol base solution.*—0.25 g KCN + 22 g 2-amino-2-methyl-1-propanol (Aldrich Chemical Co., Milwaukee, WI 53233) diluted with water to 100 mL. Store in polyethylene container.

(c) *Cresolphthalein-base working reagent.*—Mix equal volumes of reagent **a** and **b** to form red solution. Store in acid-washed glass bottle. (Glassware used for preparation of this solution must be washed with dilute nitric acid to remove any trace amounts of calcium. Rinsing all glassware with working reagent ensures freedom from contamination.)

(d) *Calcium standard solutions.*—1–5 ppm. Prepare 1000 ppm stock solution by adding 2.4970 g calcium carbonate primary standard in 2 L beaker with 50 mL water. Add 7 mL HCl dropwise and boil solution 3–4 min to remove carbon dioxide. Transfer to 1 L volumetric flask washed by dilute nitric acid, and dilute with water. Prepare working solutions by appropriate dilutions with water.

(*4*) *Iron.*

(a) *Ferrozine reagent.*—Dissolve 0.080 g Ferrozine iron reagent (Hach Chemical Co. Loveland, CO 80537) in 100 mL 2M acetate buffer, pH 4.5.

(b) *Ascorbic acid reagent.*—1% in water.

(c) *Iron(III) standard solutions.*—0.5–10 ppm. Prepare 1000 ppm stock solution by dissolving 1.000 g analytical grade iron wire (99.88%) with aqua regia (3 mL HNO_3 + 9 mL HCl). Add 3 mL $HClO_4$ and evaporate solution almost to dryness. Dilute to 1 L with water. Prepare working standard solutions by appropriate dilutions with 0.3M $HClO_4$ (so that standards are in same matrix as samples after digestion).

(d) *Blank digest solution.*—0.3M $HClO_4$.

(*5*) *Magnesium.*

(a) *Calmagite reagent.*—Dissolve 0.020 g calmagite (Sigma) and 7.46 g KCl in 100 mL water. Store in polyethylene container.

(b) *Base solution.*—1 g KCN, 4.964 g KOH, and 0.700 g EGTA (Sigma)/L water. Store in polyethylene container.

(c) *Calmagite-base working solution.*—Equal volumes of reagents **a** and **b**. Prepare fresh daily and store in polyethylene bottle closed when not in use.

(d) *Magnesium standard solutions.*—0.2–2 ppm. Prepare 1000 ppm stock solution by dissolving 8.4485 g $MgCl_2.6\ H_2O$ (99%) in 1 L water. Prepare working standard solutions by appropriate dilutions with water.

(e) *Methyl red indicator solution.*—0.2 g/100 mL alcohol.

(f) *Sodium hydroxide.*—0.1N.

Digestion

(a) *Crude protein and phosphorus determination.*—Digest samples as described in official method **7.031** (19), using reagent *1*(e).

(b) *Calcium, iron, and magnesium determinations.*—Digest samples with HNO_3–$HClO_4$ as described in official method **7.097** (19), using 2.5 g feed samples. Be sure that NO_2 fumes have been driven off, because nitrites in high concentration interfere with iron-ferrozine procedure.

Procedures

(*1*) *Crude protein.*—Put 630 nm filter in photometer module of analyzer and load reaction rate routine program from cassette recorder into computer memory. Using dispenser/diluter, mix 1 mL blank digest solution *1*(g), each nitrogen standard, and digested samples with 4.00 mL phenol reagent *1*(a) in 5 mL polyethylene cups and load them on turntable. Use one channel of stopped-flow analyzer to aliquot hypochlorite-buffer reagent *1*(d) and the other to aliquot standards and samples. During program run,

Table 1. Typical nitrogen results, using reaction-rate method [a]

Std, mg N/250 mL [b]	Rate, mA/s [c]	RSD, %
12.5	7.01	0.6
25.0	11.50	0.8
37.5	16.36	0.7
50.0	20.76	0.8
62.5	26.52	0.8
75.0	31.64	0.4

[a] Working curve: slope = 0.394, intercept = 1.70, r = 0.9991.
[b] Final concentration in observation cell is the 1/8.
[c] Average of 4 determinations on a single standard with 10-s delay and 15-s measurement time. λ = 630 nm.

Table 2. Typical phosphorus results, using reaction rate method [a]

Std, ppm P [b]	Rate, mA/s [c]	RSD, %
20.0	26.90	0.2
40.0	47.65	0.2
60.0	70.04	0.5
80.0	91.63	0.4
100.0	113.96	0.3

[a] Working curve: slope = 1.091, intercept = 4.60, r = 0.99992.
[b] Final concentration in observation cell is the 1/8.
[c] Average of 5 determinations on a single standard with 2-s delay time and 4-s measurement time. λ = 650 nm.

0 and 100% transmittances are set using reagent and blank, and operator is asked time parameters, delay and measurement time, number of flushes to change from sample to sample, number of measurements to be averaged, number and concentration of standards, and number of samples to be analyzed. Set delay time of 10 s, measurement time of 15 s, 4 flushes, and 3 measurements. The analyzer sequences through standards and calculates calibration curve, each sample is measured, and value is printed. Use mg N/250 mL found for each sample to determine percent protein nitrogen, using following equation:

% Protein = (mg N/250 mL) × (6.25/mg sample) × 100

(2) *Phosphorus.*—Use 650 nm filter. Mix 1 mL blank digest solution *1*(**g**) and each standard and sample with 4.00 mL ascorbic acid reagent *2*(**b**). Use molybdenum reagent *2*(a) and proceed as in crude protein procedure, using delay time of 2 s and measurement time of 4 s. Calculate percent phosphorus from ppm P found for each sample, using following equation:

% P = [(ppm P/4)/(mg sample)] × 100

(3) *Calcium.*—Put 580 nm filter in photometer and load equilibrium routine program in computer. Dilute samples with water to 1–5 ppm range. (For samples analyzed in this work, dilutions of 1/25, 1/50, and 1/100 were required.) Use deionized water as blank and cresolphthalein-base working reagent 3(**c**). Set delay time of 5 s and measurement time of 1 s (two 0.5-s integrations). From ppm Ca found for each sample and dilution factor D, calculate percent calcium from following equation:

% Ca = (ppm Ca/4) × (D/mg sample) × 100

(4) *Iron.*—Use 560 nm filter. Dilute samples to 0.5–10 ppm range using 0.3M $HClO_4$ (so standards and all samples will be in same matrix). Samples analyzed in this work required dilutions of 1/2 or 1/5. Mix 2 mL blank digest *4*(**d**) and each standard or sample with 2 mL ascorbic acid reagent *4*(**b**). Use equilibrium routine program with 10-s delay and 2.5-s measurement time (5 integrations). Use equation similar to calcium equation to calculate percent Fe for each sample.

(5) *Magnesium.*—Use 520 nm filter. Dilute samples with water, using 50 mL volumetric flasks and at the same time roughly neutralize with 0.1N NaOH, using 2 drops of methyl red indicator (change from red to yellow). Samples in this work required 1/25 or 1/50 dilutions. Use equilibrium program with 5-s delay and 2.5-s measurement time (5 integrations). Calculate percent Mg for each sample, using equation similar to calcium equation.

Measurements for all procedures were carried out in an air-conditioned laboratory maintained at a nominal temperature of 25°C.

Results and Discussion

The reaction-rate procedures for the determination of protein nitrogen and phosphorus described earlier (*7*, *8*) were used in this work without any modification. Because the pH of the measured solution in the observation cell is an important parameter in these reaction-rate determinations, the variation in the sulfuric acid concentration in the digested samples was examined by titration with NaOH standard solution. The acid remaining was 47 ± 6% of the original. This loss is overcome by preparing the nitrogen and phosphate standards in the same matrix as the samples after digestion.

Typical results for working curves are shown in Tables 1 and 2 for crude protein and phosphorus, respectively, and demonstrate the excellent precision and linearity obtained for the standards.

Table 3. Typical calcium results, using equilibrium method [a]

Std, ppm Ca [b]	Absorbance [c]	RSD,%
1.00	0.159	0.7
2.00	0.354	0.2
3.00	0.615	0.2
4.00	0.837	0.1
5.00	1.072	0.1

[a] Working curve: slope = 0.231, intercept = −0.085, r = 0.9993.
[b] Final concentration in cell is the 1/2.
[c] Average of 5 determinations on a single standard with 5-s delay and 1-s measurement time (2 integrations). λ = 580 nm.

Table 5. Typical iron results, using equilibrium method [a]

Std, ppm Fe [b]	Absorbance [c]	RSD, %
0.500	0.056	1.0
1.00	0.118	0.4
5.00	0.588	0.1
7.50	0.892	0.2
10.00	1.176	0.1

[a] Working curve: slope = 0.118, intercept = −0.001, r = 0.99996.
[b] Final concentration in observation cell is the 1/4.
[c] Average of 5 determinations on a single standard with 10-s delay and 2.5-s measurement time (5 integrations). λ = 560 nm.

The reaction of calcium with *o*-cresolphthalein complexone is complete in less than the dead time of the stopped-flow unit (200 ms) so that an equilibrium procedure with a short delay time can be performed. A delay time of 5 s was chosen to avoid nonreproducible absorbance behavior noted when the working reagent is mixed with the standard and sample solutions. A measurement time of 1.0 s (two 0.5-s integrations) gave excellent precision. The cresolphthalein complexone concentration (0.147 mM final) was chosen as a compromise of low absorbance blank and good linearity of the working curve. Final concentrations of 1.5 mM for 8-quinolinol and 9.6 mM for CN^- ensure elimination of magnesium and heavy metals interference. Because of the relatively high concentration of calcium in feeds, an extensive dilution of the sample digest solutions is required. The acid concentration of the diluted samples is low and a 0.62M aminomethyl propanol concentration ensures pH stability. Typical results for the calcium working curve are shown in Table 3, again with good precision and linearity.

The reaction of iron (II) with ferrozine is also rapid and an equilibrium procedure is proposed.

Table 4. Iron results using Fe(II) and Fe(III) standards

	Absorbance	
Std, ppm Fe [a]	Fe(II)	Fe(III)
0.25	0.068	0.068
0.50	0.119	0.125
1.00	0.233	0.243
3.00	0.723	0.719
5.00	1.195	1.194
Slope	0.239	0.237
Intercept	0.003	0.006
Corr. coeff. (r)	0.99994	0.999994

[a] In 0.3M $HClO_4$ and 1% ascorbic acid solution.

A small increase in the absorbance is noted during the first few seconds of the reaction, so a 10-s delay time was used to ensure stability of the absorbance values. A mixed ferrozine–ascorbic acid reagent was tried with good results for aqueous standard Fe(III) solutions but with a little lower results for samples. So the preaddition of ascorbic acid in the samples was preferred. The efficiency of the iron(III) reduction with ascorbic acid was checked by obtaining results using iron(II) and iron(III) standards. The results as shown in Table 4 are similar. A measurement time of 2.5 s (5 successive 0.5-s integrations) gave the best precision. The standards and the dilutions of the sample digest solutions are made with 0.3M $HClO_4$, which is the average concentration of the digest solutions, to ensure matrix similarity of samples and standards. The ferrous complex of ferrozine is stable in the pH range 4–10 (12) so an acetate buffer pH 4.5 with a 1M final concentration was chosen. Typical results for iron calibration curves are shown in Table 5.

The magnesium–calmagite reaction is also fast and an equilibrium procedure with 5-s delay

Table 6. Typical magnesium results, using equilibrium method [a]

Std, ppm Mg [b]	Absorbance [c]	RSD, %
0.200	0.070	0.3
0.500	0.189	0.6
1.00	0.364	0.4
1.50	0.539	0.2
2.00	0.690	0.2

[a] Working curve: slope = 0.345, intercept = 0.012, r = 0.9992.
[b] Final concentration in observation cell is 1/2.
[c] Average of 5 determinations on a single standard with 5-s delay and 2.5-s measurement time (5 integrations). λ = 520 nm.

Table 7. Comparison of reaction-rate stopped-flow method with official AOAC method for determining crude protein

Sample	Crude protein, %			
	Stopped-flow[a]	Official[b]		Diff.,[c] %
Swine feed (7828)	41.63 ± 0.20	40.77 ± 0.46	D	+0.86
Pig feed (7829)	18.96 ± 0.12	18.38 ± 0.26	D	+0.58
Poultry ration (7831)	19.68 ± 0.05	18.44 ± 0.30	D	+1.24
Soybean meal (7921)	46.28 ± 0.35	45.83 ± 0.68	D	+0.45
Dairy ration (7922)	19.05 ± 0.26	18.38 ± 0.28	D	+0.67
Dried molasses (7923)	10.81 ± 0.06	10.48 ± 0.20	D	+0.33
Swine feed (7924)	22.64 ± 0.09	22.14 ± 0.29	D	+0.50
Milk replacer (7925)	20.49 ± 0.13	20.50 ± 0.26	D	−0.01
Beef cattle sup. (7926)	56.94 ± 0.12	55.27 ± 0.49	D	+1.67
Swine feed (7928)	15.89 ± 0.12	15.76 ± 0.23	D	+0.13
Pig feed (7929)	17.24 ± 0.04	17.07 ± 0.27	D	+0.17
Beef feed (7930)	44.06 ± 0.30	44.85 ± 0.57	D	−0.79
Chick starter (7931)	19.38 ± 0.12	20.06 ± 0.27	D	−0.68
Swine feed (7932)	14.94 ± 0.14	14.38 ± 0.25	D	+0.56
Dairy feed (8021)	43.42 ± 0.34	43.49 ± 0.56	K	−0.07
Dairy feed (8022)	33.70 ± 0.19	34.05 ± 0.46	K	−0.35
Broiler ration (8025)	20.59 ± 0.12	20.09 ± 0.26	K	+0.50
Dried molasses–$NaNO_3$ (8024)	12.29 ± 0.08	12.39 ± 0.39	K	−0.10
Swine feed (8025)	20.82 ± 0.10	18.99 ± 0.30	K	+1.83
Cattle sup. (8027)	39.29 ± 0.14	39.63 ± 0.55	K	−0.34

[a] Average of 4 determinations performed on single digestion of check feed ±1 SD.
[b] Values reported in AAFCO Check Feed Sample Program by 74–90 laboratories during 1978–80, ±1 SD. Official methods **7.015** (20) (Kjeldahl method (K)), and **7.016** (Dumas method (D)).
[c] Stopped-flow average − official method average.

time and 2.5-s measurement time gave excellent precision. The calcium interference was checked with mixed magnesium–calcium standards. The EGTA concentration used (0.46 mM final) eliminated any interference up to 1:10 concentration ratio. A 0.25M final KCl concentration provides ionic-strength similarity of standards and samples. A rough neutralization of the acid-digested samples is required to keep the solution at the desired pH with the 0.022M

Table 8. Comparison of reaction-rate stopped-flow method with official AOAC method for determining phosphorus

Sample[a]	Phosphorus, %		
	Stopped-flow[b]	Official[c]	Diff.,[d] %
7828	1.944 ± 0.024	1.925 ± 0.094	+0.019
7829	0.620 ± 0.003	0.626 ± 0.031	−0.006
7831	0.634 ± 0.006	0.628 ± 0.035	+0.006
7921	0.659 ± 0.006	0.639 ± 0.034	+0.020
7922	0.863 ± 0.004	0.799 ± 0.055	+0.064
7923	0.101 ± 0.001	0.129 ± 0.018	−0.028
7924	0.690 ± 0.008	0.678 ± 0.031	+0.012
7925	0.837 ± 0.003	0.833 ± 0.040	+0.004
7926	1.295 ± 0.009	1.251 ± 0.061	+0.044
7928	0.594 ± 0.004	0.584 ± 0.030	+0.010
7929	0.769 ± 0.006	0.735 ± 0.035	+0.034
7930	1.090 ± 0.005	1.114 ± 0.063	−0.024
7931	0.782 ± 0.005	0.793 ± 0.046	−0.011
7932	0.628 ± 0.005	0.622 ± 0.033	+0.006
8021	1.395 ± 0.012	1.425 ± 0.052	−0.030
8022	1.160 ± 0.007	1.143 ± 0.047	+0.017
8023	0.766 ± 0.003	0.758 ± 0.029	+0.008
8024	0.078 ± 0.004	0.088 ± 0.016	−0.010
8025	0.774 ± 0.003	0.773 ± 0.035	+0.001
8027	1.280 ± 0.011	1.281 ± 0.051	−0.001

[a] Samples identified in Table 7.
[b] Average of 5 determinations performed on single digestion of check feed ±1 SD.
[c] Values reported in AAFCO Check Feed Sample Program by 19–56 laboratories during 1978–80, ±1 SD. Official method, **7.120** (20) (molybdovanadate photometric method).
[d] Stopped-flow average − official method average.

Table 9. Comparison of stopped-flow method with official AOAC method for determining calcium

Sample[a]	Calcium, %		
	Stopped-flow[b]	Official[c]	Diff.,[d] %
7828	3.993 ± 0.007	3.859 ± 0.289	+0.134
7829	0.763 ± 0.002	0.737 ± 0.058	+0.026
7831	0.802 ± 0.001	0.882 ± 0.042	−0.080
7921	0.303 ± 0.002	0.297 ± 0.016	+0.006
7922	1.018 ± 0.002	1.042 ± 0.075	−0.024
7923	0.724 ± 0.002	0.783 ± 0.043	−0.059
7924	0.887 ± 0.002	0.854 ± 0.042	+0.033
7925	0.863 ± 0.002	0.820 ± 0.067	+0.043
7926	2.594 ± 0.004	2.594 ± 0.148	0.000
7928	0.747 ± 0.002	0.711 ± 0.063	+0.036
7929	0.995 ± 0.001	1.039 ± 0.084	−0.044
7930	6.128 ± 0.011	6.292 ± 0.403	−0.164
7931	0.941 ± 0.001	0.992 ± 0.067	−0.051
7932	0.810 ± 0.003	0.802 ± 0.038	+0.008
8021	0.893 ± 0.001	0.982 ± 0.062	−0.089
8022	4.468 ± 0.007	4.524 ± 0.207	−0.056
8023	1.096 ± 0.003	1.113 ± 0.079	−0.017
8024	0.430 ± 0.001	0.451 ± 0.037	−0.021
8025	0.811 ± 0.001	0.864 ± 0.077	−0.053
8027	4.637 ± 0.011	4.439 ± 0.273	+0.198

[a] Samples identified in Table 7.
[b] Average of 5 determinations performed on single digestion of check feed ±1 SD.
[c] Values reported in AAFCO Check Feed Sample Program by 16–55 laboratories during 1978–80, ±1 SD. Official method, **7.091** (20) (atomic absorption spectrophotometry).
[d] Stopped-flow average − official method average.

final concentration of KOH. The yellow methyl red indicator does not interfere. Addition of Bion NE-9 and Bion PVP (16) in the calmagite reagent, which prevents the shifting by serum proteins of the maximum of the absorption spectrum of Mg–calmagite complex, showed no effect in the analysis of digested feeds. Typical results for magnesium working curves are shown in Table 6.

To evaluate the accuracy of the proposed

Table 10. Comparison of stopped-flow method with official AOAC method for determining iron

Sample[a]	Iron, %		
	Stopped-flow[b]	Official[c]	Diff.,[d] %
7828	0.0828 ± 0.0002	0.0900	−0.0072
7829	0.0347 ± 0.0002	0.0365 ± 0.0040	−0.0018
7831	0.0225 ± 0.0002	0.0288 ± 0.0029	−0.0063
7921	0.0165 ± 0.0004	0.0178 ± 0.0044	−0.0013
7922	0.0993 ± 0.0004	0.1171 ± 0.0085	−0.0176
7923	0.0524 ± 0.0002	0.0536 ± 0.0047	−0.0012
7924	0.0462 ± 0.0007	0.0539 ± 0.0208	−0.0077
7925	0.0161 ± 0.0001	0.0180 ± 0.0019	−0.0019
7926	0.1051 ± 0.0010	0.1025 ± 0.0167	+0.0026
7928	0.0233 ± 0.0001	0.0240 ± 0.0012	−0.0007
7929	0.0333 ± 0.0003	0.0343 ± 0.0018	−0.0010
7930	0.1412 ± 0.0020	0.1561 ± 0.0166	−0.0149
7931	0.0220 ± 0.0003	0.0235 ± 0.0024	−0.0015
7932	0.0309 ± 0.0001	0.0307 ± 0.0045	+0.0002
8021	0.0597 ± 0.0002	0.0582 ± 0.0039	+0.0015
8022	0.0581 ± 0.0003	0.0572 ± 0.0042	+0.0009
8023	0.0314 ± 0.0001	0.0312 ± 0.0043	+0.0002
8024	0.0344 ± 0.0002	0.0348 ± 0.0051	−0.0004
8025	0.0281 ± 0.0001	0.0268 ± 0.0036	+0.0013
8027	0.0693 ± 0.0001	0.0706 ± 0.0049	−0.0013

[a] Samples identified in Table 7.
[b] Average of 5 determinations performed on single digestion of check feed ±1 SD.
[c] Values reported in AAFCO Check Feed Sample Program by 1–8 laboratories during 1978–80, ±1 SD. Official method, **7.077** (20) (atomic absorption spectrophotometry).
[d] Stopped flow average − official method average.

Table 11. Comparison of stopped-flow method with official AOAC method for determining magnesium

Sample[a]	Magnesium, %		
	Stopped-flow[b]	Official[c]	Diff.,[d] %
7828	0.2774 ± 0.0015	0.2745 ± 0.0158	+0.0029
7829	0.1385 ± 0.0007	0.1439 ± 0.0105	−0.0054
7831	0.1472 ± 0.0013	0.1314 ± 0.0073	+0.0158
7921	0.3019 ± 0.0013	0.2824 ± 0.0276	+0.0195
7922	0.3615 ± 0.0015	0.3508 ± 0.0162	+0.0107
7923	0.2708 ± 0.0011	0.2808 ± 0.0427	−0.0100
7924	0.1961 ± 0.0006	0.1953 ± 0.0143	+0.0008
7925	0.1230 ± 0.0004	0.1245 ± 0.0070	−0.0015
7926	0.3543 ± 0.0003	0.3397 ± 0.0405	+0.0146
7928	0.2354 ± 0.0007	0.2398 ± 0.0202	−0.0044
7929	0.2259 ± 0.0009	0.2218 ± 0.0169	+0.0041
7930	0.5281 ± 0.0005	0.5236 ± 0.0421	+0.0045
7931	0.2429 ± 0.0009	0.2347 ± 0.0273	+0.0082
7932	0.2236 ± 0.0010	0.2124 ± 0.0169	+0.0112
8021	0.4055 ± 0.0003	0.4007 ± 0.0286	+0.0048
8022	0.3770 ± 0.0001	0.3582 ± 0.0301	+0.0188
8023	0.1862 ± 0.0010	0.1740 ± 0.0278	+0.0122
8024	0.1092 ± 0.0004	0.1151 ± 0.0416	−0.0059
8025	0.2088 ± 0.0010	0.2218 ± 0.0157	−0.0130
8027	0.4177 ± 0.0009	0.4350 ± 0.0499	−0.0173

[a] Samples identified in Table 7.
[b] Average of 5 determinations performed on single digestion of check feed ±1 SD.
[c] Values reported in AAFCO Check Feed Sample Program by 3–12 laboratories during 1978–80, ±1 SD. Official method, **7.077** (20) (atomic absorption spectrophotometry).
[d] Stopped-flow average − official method average.

methods, a series of samples assayed by official methods by other laboratories were analyzed; see Tables 7–11. The data demonstrate that the accuracy of the proposed methods is within the reported uncertainty of the official procedures. There is a small positive bias on the determination of protein.

Statistically summarized data of the comparison are shown in Table 12. All the methods show excellent correlation, with slopes of 0.9063–1.0089 and correlation coefficients of 0.9929–0.9989.

Table 12. Regression equations for comparison of stopped-flow (SF) methods with official AOAC (20) methods (20 samples)

Y(SF) (range)	X(AOAC)	Slope	Intercept (%)	Corr. coeff. (r)
Crude protein (10.8–56.9%)	**7.015** (Kjeldahl) **7.016** (Dumas)	0.9992	0.38	0.9987
Phosphorus (0.078–1.94%)	**7.120** (photometric)	1.0089	−0.001	0.9986
Calcium (0.30–4.64%)	**7.091** (AAS)	1.0023	−0.012	0.9989
Iron (0.016–0.141%)	**7.077** (AAS)	0.9063	0.0021	0.9929
Magnesium (0.109–0.528%)	**7.077** (AAS)	1.0048	0.0023	0.9955

Using 4 flushes to change from one solution to another (flush cycle time about 1.5 s), one measurement per sample and assuming 1 s for the turntable position increment, and 0.5 s for the computer calculation and printing time, the analysis rates shown in Table 13 can be obtained by the methods reported. The total sample or reagent volume consumed is 0.75 mL.

The proposed methods reported here provide rapid, precise, and accurate determination of protein, phosphorus, calcium, magnesium, and

Table 13. Routine analysis rates

Detn	Anal. rate (samples/h)	Delay time, s	Measurement time, s
Crude protein (reaction-rate)	110	10	15
Phosphorus (reaction-rate)	266	2	4
Calcium (equilibrium)	266	1	5
Iron (equilibrium)	180	2.5	10
Magnesium (equilibrium)	240	2.5	5

iron and have the distinct advantages of high sample throughput and utilization of only one relatively simple automated system.

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TECHNICAL COMMUNICATIONS

Elimination of Sodium Chloride Interference During High Pressure Liquid Chromatographic Determination of Sugars

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A method is described to eliminate the potential interference of sodium chloride found in some food samples, which occurs during high pressure liquid chromatographic (HPLC) analysis of sugars, using certain bonded aminopropyl columns. The column is tested, and if an interference is present, it is removed by washing the column with a solution of tetraethylenepentamine in mobile phase. HPLC separation and quantitation of the sugars are essentially the same as before washing; however, sodium chloride no longer interferes with any of the sugars being analyzed.

In 1979, our laboratories reported the results of a study of a high pressure liquid chromatographic (HPLC) method for the analysis of sugars in food samples (1). We have used this method routinely for the quantitation of sugars since that time. When some (but not all) carbohydrate (Waters Associates, Milford, MA) and propylamino (E. Merck, Darmstadt, GFR) columns are used, a peak other than the solvent front and the normal mono- and disaccharides being analyzed appears in the chromatogram. In some cases, this peak does not interfere with quantitating the sugars in the sample, but often the peak elutes at approximately the same time as fructose or glucose (Figures 1 and 2). The occurrence and size of this peak led us to believe that it might be sodium chloride. This was confirmed by injecting a solution of sodium chloride under the same conditions used for sugar analysis (Figure 2).

Adding a column modifier, such as a polyamine, should affect the interaction of ionic species with the column more than it would affect the interaction of the carbohydrates and, therefore, should resolve the interfering peak and the fructose and glucose peaks. This proved to be the case. Washing the column with a solution of 0.1% tetraethylenepentamine (TEPA) in mobile phase, and then returning to the normal mobile phase eliminates the interference by the sodium chloride peak. Sodium chloride apparently elutes as the negative peak just after the solvent front (Figure 3), and the retention times of the sugars in the chromatogram are not changed dramatically (Figure 4). We have found in our laboratories that several hundred samples can be analyzed before the column needs additional treatment.

A number of collaborative studies have been carried out on HPLC analysis of sugars. Two AOAC official first action methods have resulted (**13.A01–13.A04** (2) and **14.C01–14.C04** (3)). Because sodium chloride represents a potential interference, we believe that the following steps should be made a part of those methods:

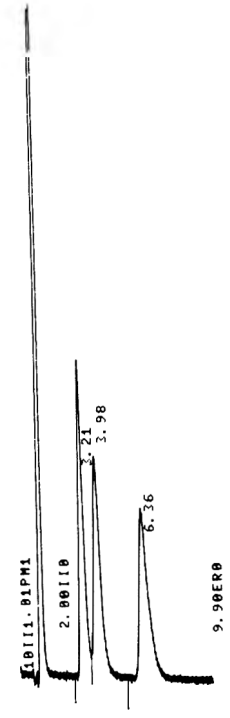

Figure 1. Chromatogram of sugar standards. R_T (min) = 3.21, fructose; 3.98, glucose; 6.36, sucrose. Full scale pen response = 4 × (188 × 10^{-6}) refractive index unit. Concentration of each sugar 4 mg/mL. See Ref. 1 for chromatographic conditions.

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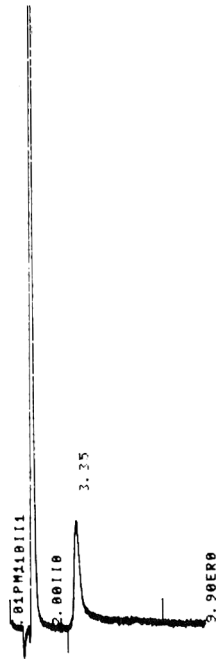

Figure 2. Chromatogram of sodium chloride, 2 mg/mL. Same conditions as in Figure 1. R_T = 3.35 min.

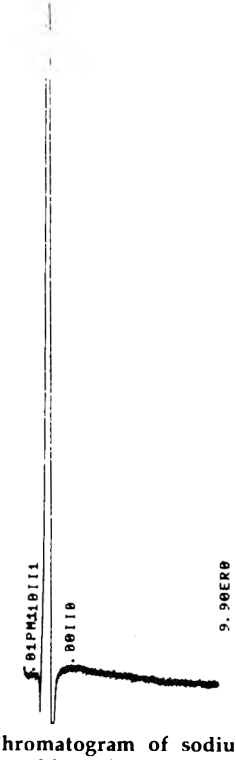

Figure 3. Chromatogram of sodium chloride, 2 mg/mL, after washing column for 2 h with 0.1% TEPA solution. Same conditions as in Figure 1.

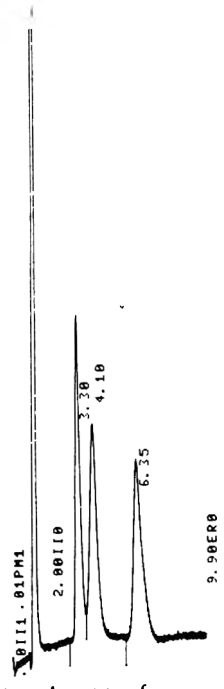

Figure 4. Chromatogram of sugar standards, after washing column for 2 h with 0.1% TEPA solution. Same conditions as in Figure 1.

(*1*) Prepare a blank solution of approximately 2 mg sodium chloride/mL in the same injection solvent as that used for the sugar standards, and inject this blank solution immediately after the sugar standards and before injecting the sample solutions.

(*2*) If the sodium chloride peak interferes with any of the peaks of the sugars being analyzed, wash the column with a solution of 0.1% TEPA (Catalog No. T5902, Eastman Kodak Co., Rochester, NY 14650) in acetonitrile–water (80 + 20). Adjust the pH of the TEPA solution to approximately 7 with acetic acid and then wash the column for 2 h at 1.5 mL/min. Flush the column with approximately 100 mL normal mobile phase before continuing the analytical series.

We believe that adding these 2 steps to methods **13.A01** and **14.C01** will eliminate erroneously high sugar results when sodium chloride has the same retention time as that for one of the sugars under the conditions of the method.

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Comparison Between Potentiometric Titration and Enzyme-Catalyzed Determination of Hydrogen Peroxide

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A titrimetric procedure has been recommended for the determination of residual amounts of hydrogen peroxide on packaging surfaces as a result of its use as a sterilant. Enzymatic assays have been described for the determination of hydrogen peroxide in various substrates. We compared the enzymatic assay based on the oxidation of leuco crystal violet by peroxidase and the potentiometric titration procedure for the determination of hydrogen peroxide in distilled water, as required in the approval of this material as a packaging sterilant. The correlation between the 2 procedures is 0.976.

Although the bactericidal action of hydrogen peroxide has been recognized for quite some time (1), the possibility of using hydrogen peroxide as a chemical sterilant for food packaging materials has been recognized only in the past few years (2). Early in 1981 (3), the Food and Drug Administration approved the use of hydrogen peroxide as a sterilant for polyethylene packaging films used in aseptic packaging systems, with the provision that the maximum residual level found when the packages are filled with distilled water would be 0.1 ppm. At the time of approval, FDA recognized the difficulty of analyzing such low levels and announced that further methodology studies were being undertaken. Subsequently, FDA recommended the procedure of Boto and Williams (4), a potentiometric titration using dilute solutions of potassium permanganate.

Prior to and then concurrent with the FDA methodology study, our laboratory had also been investigating methodology suitable for the determination of low levels of hydrogen peroxide. In addition to the potentiometric method (4), numerous other electrochemical procedures (5, 6) are available for determining hydrogen peroxide.

An electrode-based procedure has also been described (7). But perhaps the simplest and most common approach has been the indirect determination of hydrogen peroxide through the measurement of the spectrometric properties of reaction products of various compounds with hydrogen peroxide. These latter procedures may involve the oxidation of transition and post-transition metal chelates (8), or the oxidation of organic compounds to give colored or fluorescent compounds (9). A limitation of most of these procedures is that reaction rates in the presence of low concentrations of hydrogen peroxide may be quite slow. An additional limitation is the possibility of either negative or positive interferences as a result of the nonspecificity of the redox reactions.

Numerous enzyme-catalyzed procedures for the determination of hydrogen peroxide have been described (10). These catalase- or peroxidase-based procedures involve the enzyme-catalyzed oxidation of an organic substrate to a species which can be detected spectrophotometrically. The enzyme-based procedures offer several important advantages for routine use. They offer high specificity and fast reaction rates even at low concentrations of hydrogen peroxide. The judicious choice of organic substrates can lead to oxidized species with high molar absorptivities with the concomitant increased sensitivity of the assay. Finally, the enzyme-catalyzed spectrophotometric assays are simple and fast to run. These factors combined with our intention to be able to apply the assay to routine monitoring applications led us to investigate a procedure first described by Mottola et al. (11) using the peroxidase-catalyzed oxidation of leuco crystal violet to determine hydrogen peroxide.

In this communication we describe the peroxidase procedure as employed in our laboratory and show data comparing results with those obtained by the recommended potentiometric titration methods.

Experimental

The methods are those originally described by Boto and Williams (4) and Mottola et al. (11). The original papers should be consulted for analytical details.

In the test of the potentiometric procedure, a platinum wire electrode was used with a standard calomel reference electrode. Electrode potentials were monitored with a digital pH/mV meter. The end point potential was determined by titrating 100 mL of a 0.3 ppm standard solu-

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tion of hydrogen peroxide with 0.001M $KMnO_4$. Potentials were recorded and manually plotted. Subsequent titrations were conducted to this end point.

For the enzymatic determination, leuco crystal violet (Aldrich Chemical Co.) was purified by dissolving the powder in dilute HCl followed by extracting the leuco dye into ethyl acetate. After the solvent was evaporated, the compound was recrystallized from ethyl acetate. Hydrogen peroxide concentrations were obtained in 5 mL aliquots of sample by adding in succession 1 mL of 0.5 mg leuco dye/mL solution and 0.5 mL of 1.0 mg horseradish peroxidase/mL solution. Five milliliters of acetate buffer (pH 4.5) was added to bring the absorption maximum to 596 nm. The concentration of hydrogen peroxide can then be determined directly from the standard curve of absorbance vs concentration of hydrogen peroxide.

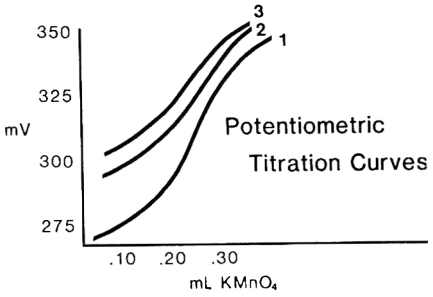

Figure 1. Three successive titrations of 50 mL 0.3 ppm H_2O_2 with 0.001M $KMnO_4$. Electrodes were stored overnight in distilled water before start of titrations.

Results and Discussion

Figure 1 shows 3 successive titration curves obtained on dilute solutions of hydrogen peroxide in distilled water. The figure shows a gradual change in the observed end point potential as the platinum electrode apparently becomes slowly conditioned. After the second or third titration, the end point potential generally stabilizes. A second observation from Figure 1 is that the inflection point of the titration curves is difficult to determine manually. The titration curve does not exhibit a sharp change in potential at the end point but rather the change extends over several volumes of titrant. These 2 phenomena are related and may be explained by the observation made by Harrar (6) that hydrogen peroxide undergoes a rapid heterogeneous catalytic decomposition at a platinum electrode. The decomposition was 99.9% complete within 6 min. This rapid reaction then competes with the permanganate oxidation reaction. The potentiometric procedure is further complicated by the fact that at the low levels of reactants involved in the titration the reaction rate would be expected to be comparatively slow. These 2 factors could combine to adversely affect the shape of the titration curve. Harrar further showed the dependence of the electrolysis time and background current during the electrolysis of hydrogen peroxide on the pre-treatment of the platinum electrode. The formation of an oxide layer on the platinum surface significantly affected the response characteristics of the electrode. It would seem likely that similar considerations would apply when measuring potentials at the platinum electrode.

In spite of the limitations cited above, if, after the electrode has become stabilized, solutions are consistently titrated to the same end point, then the procedure will yield reproducible results. The standard curve plot of volume of titrant vs hydrogen peroxide concentration is linear over the range of interest, up to 0.20 ppm. The standard curve however exhibits a negative y-axis intercept which is consistent with the existence of an alternative pathway for the disappearance of hydrogen peroxide.

In the enzymatic determination, peroxidase catalyzes the oxidation of leuco crystal violet by hydrogen peroxide. The absorbance of the solution follows Beer's Law. The standard curve plot of absorbance vs hydrogen peroxide concentration is linear through 1.0 ppm. Like the standard curve of the potentiometric procedure, the standard curve exhibits a negative intercept on the y-axis, suggesting the presence of a competitive reaction. Mottola et al. (11) have suggested that the sensitivity of the enzymatic procedure is near maximum, based on the molar absorptivity of crystal violet. The enzyme-catalyzed reaction is very rapid; color develops immediately upon addition of the enzyme. Although the previous authors stated that absorbance readings for the blank showed no change for several hours, in our hands the color of both the assay and blank solutions deepened on prolonged standing. We took no extraordinary precautions to exclude light or air from the solutions once mixed because, as long as at least 2 standard solutions were analyzed at the same time as the unknowns, the assay did not suffer in accuracy.

The results of successive analyses of solutions of distilled water fortified at 2 levels are shown

Table 1. Results of repetitive analysis of water fortified with H_2O_2

	0.16 ppm		0.10 ppm	
	Enz.	Pot.	Enz.	Pot.
	0.16	0.20	0.11	0.10
	0.16	0.22	0.11	0.10
	0.17	0.19	0.10	0.11
	0.17	0.17	0.11	0.10
	0.16	0.19	0.12	0.10
	0.16	0.22	0.10	0.10
	0.17	0.19	0.11	0.10
	0.16	0.19	0.10	0.11
	0.18	0.17	—	—
$\bar{x}$	0.17	0.19	0.11	0.10
s	0.007	0.018	0.007	0.004
CV, %	4.4	9.3	6.6	4.3

in Table 1. At the 2 levels examined, there appears to be no difference in repeatability as measured by the coefficient of variation (CV). The CV values at these levels are quite acceptable. Table 2 continues the comparison between results obtained with the 2 methods studied. The correlation coefficient between results from the 2 methods was 0.976.

As seen in Table 2 the absolute differences between observed and actual peroxide concentration ranges from 0.01 to 0.03 ppm. For the potentiometric method, this represents 0.02–0.06 mL titrant. Attempts to increase the sensitivity of the assay by decreasing the concentration of the titrant and thus increasing the amount required to reach the end point were not successful because of the slowness of the reaction as a result of the decrease in concentration of reactants. The enzymatic assay, however, was fairly insensitive to changes in concentration of enzyme and leuco dye. A reduction in the added amount of peroxidase solution by one-fifth slows the rate of color formation but has no other effect on the determination. The amount of leuco crystal violet added may be reduced by one-half with no effect on the assay of solutions containing between 0.02 and 1.0 ppm hydrogen peroxide. Although no attempts were made to increase the sensitivity of the enzymatic assay, such an increase could be practically obtained by decreasing the amount of buffer solution used. In as much as the assay solutions consist of distilled water, this change in the concentration of buffer in the final solution should be negligible.

The results discussed above indicate that the potentiometric procedure of Boto and Williams (4) and the enzymatic procedure described by Mottola et al. (11) will give comparable results when applied to the analysis of dilute solutions of hydrogen peroxide in distilled water. Certain considerations, however, would suggest that the enzymatic procedure would have advantages for use in routine assays. In the absence of electronics capable of calculating the first derivative of the titration curve, the end point of the potentiometric titration is difficult to determine accurately. Even when the end point potential is accurately determined, the small incremental volumes of titrant used combined with the response characteristics of the electrode tend to tax the ability of the analyst to perform accurate repetitive analyses. In contrast, the enzymatic procedure is simple and rapid. Once the reagent solutions are mixed, any convenient number of samples can be analyzed within 15 min. Due to the specificity of the enzyme-catalyzed reaction, the procedure can be extended to the detection of hydrogen peroxide in non-distilled water.

We have applied the enzymatic procedure to the measurement of hydrogen peroxide in water

Table 2. Correlation between methods (replicate determinations)[a]

H_2O_2 added, ppm	Enz.	Pot.
0.07	0.07	0.07
0.10	0.11	0.10
0.16	0.17	0.19
0.20	0.21	0.20

[a] $r = 0.976$.

exposed to packages treated with the sterilant. No interferences were encountered from materials arising from the treated polyethylene film.

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Problems in Collection of Representative Samples for Determination of Tributoxyethyl Phosphate in Potable Water

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Results of a brief investigation on tributoxyethyl phosphate in tap water are presented to illustrate the difficulties in obtaining representative samples and to alert analysts to the importance of designing suitable sampling protocols.

Tributoxyethyl phosphate (TBEP) is used as a flame retardant plasticizer in many products, e.g., synthetic rubbers and adhesives, and has been identified as a contaminant in purified water (1), potable water (2, 3), and blood samples (4, 5). Methods for determination of TBEP (2) have emphasized, in the method procedure, the avoidance or replacement of materials and chemicals that could contribute TBEP to the sample. However, during collection of potable water samples, it is not desirable to replace all TBEP-containing rubber washers/gaskets before sampling from a tap because the resultant samples then might not be representative of the water normally ingested by the consumer. Results of a brief investigation on TBEP in tap water are presented to illustrate the difficulties in obtaining representative samples and to alert analysts to the importance of designing suitable sampling protocols.

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Experimental

Reagents and Apparatus

Use solvents, chemicals, and equipment as previously described (1, 2).

Sample Collection

Grab samples were collected in pre-cleaned 4 L amber glass bottles. Raw water samples were collected at the intake bay of the treatment plant. For tap water samples, the first 2 or 4 L water was collected and then the tap was allowed to flow at 1 L/min; subsequent 2 or 4 L samples were collected at selected times.

Extractions

(a) Large-volume sampling and extraction were carried out using the XAD-2 macroreticular resin method (1, 2).

(b) Grab samples were extracted with methylene chloride as previously described (1).

(c) Rubber O-rings and seals were extracted as previously described (2).

Results and Discussion

During the development of a method of analysis for trialkyl/triaryl phosphates (TAAP) in potable water, highly variable levels of TBEP were detected in water sampled from a labora-

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Table 1. Tributoxyethyl phosphate levels (ng/L) in water samples

Water source	XAD-resin (vol. sampled)	Grab sample[a] 0–4 L	Grab sample[a] 60–64 L
River water-raw	42.9 ± 2.7 (40 L)[b]		
Treatment plant-raw tap	13.7 ± 2.6 (166 L)[c]		
Pumping station-treated tap	13.8 ± 1.0 (200 L)[c]		
Laboratory tap		5400[d]	250
Laboratory tap	25.9 ± 2.1 (195 L)[c]	433[d]	18.2
Kitchen tap-A[e]		34.9[f]	14.3
Kitchen tap-B[e]		11.8[f]	11.0
Kitchen tap-C[e]		28.1[f]	16.3

[a] Single grab samples.
[b] Duplicate samples.
[c] Triplicate samples.
[d] Sample taken after non-use of tap for 65 h.
[e] Private residence.
[f] Sample taken after non-use of tap for 16 h.

tory tap. Grab samples of water from this tap, after a weekend of non-use, showed very high TBEP levels (Table 1) that decreased as the tap was flushed with water. The O-ring and seal in the tap were identified as the source of the TBEP and were shown to contain high levels (mg/g) of TBEP (2). After replacement of the O-ring and seal with others having low (<10 μg/g) TBEP content, water levels of TBEP were greatly reduced, although there was still a slight elevation of TBEP levels in water standing in the tap for a prolonged period (Table 1). This was attributed to diffusion of TBEP from adjacent unmodified taps connected in series along the bench.

Potable water grab samples collected from 2 private residences showed increased levels of TBEP in the first-draw samples; a third residence did not show higher TBEP levels. Flushing the taps with 60 L water decreased the TBEP levels to those found in raw water at the treatment plant and in treated water at a pumping station in the distribution system (Table 1).

A recent survey (6) of drinking water consumption in Canada indicated that about 17% of consumers seldom or never flushed taps before drawing water for beverages or food preparation. Therefore, to provide appropriate data for estimating exposure to certain organic compounds, it is necessary to analyze both first draw and well flushed tap water samples. This has been considered in surveys for some inorganic contaminants (7,8), but analysts designing potable water surveys for organic contaminants should also recognize the importance of providing similar instructions in their sampling protocol.

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Treatment of Freshly Harvested 1980 Georgia Dent Corn Samples Collected for Aflatoxin Analysis

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In 1980, corn was harvested from six 15-ft rows in each of 67 fields in Georgia for aflatoxin analysis. Every sixth ear from each field was placed in a sample bag to be dried the day of collection. The rest of the corn was husked and shipped to Peoria in cardboard boxes. When undried ear samples arrived in Peoria, each sample was randomly separated into 5 equivalent subsamples. One set of 67 subsamples was shelled and dried as soon as possible to avoid further aflatoxin formation. Two other sets of 67 subsamples were stored 3 and 6 weeks before shelling and drying. The remaining 2 sets of ear samples were placed in plastic bags with 5% Monoprop (1 part propionic acid plus 1 part versite) and stored 3 and 6 weeks before shelling and drying. The samples dried in Georgia before shipping had an average total aflatoxin level of 217 ng/g. Samples shelled and dried immediately after arrival had an average level of 202 ng/g. Samples shelled and dried after 3 and 6 weeks of storage had average total aflatoxin levels of 417 and 387 ng/g, respectively. Samples stored 3 and 6 weeks in the presence of 5% Monoprop (2.5% propionic acid) had average total aflatoxin levels of 120 and 157 ng/g, respectively.

An Ad Hoc Work Group was organized in 1979 under the auspices of the U.S. Department of Agriculture to examine and summarize current knowledge regarding mycotoxin surveys, sampling techniques, conditions conducive to post-collection production of mycotoxins in grain samples, and analytical methods for mycotoxin analysis (1). The emphasis of the study was on corn suspected of containing aflatoxin. One report indicated an increase in aflatoxin concentration of approximately 10-fold (from 200 to 2300 ng/g) in a 3-day period when a field-harvested corn sample was stored at high moisture. The Ad Hoc Work Group, therefore, recommended that corn samples be dried as soon as possible after collection to moisture levels of 13–13.5% or cooled rapidly to 0°C to prevent mold growth and metabolite formation.

To determine the validity of this recommendation, freshly harvested dent corn was collected at maturity from 57 fields in Georgia in the crop year 1979 (2). The husked corn was randomly separated into 2 sets of 57 matched samples. One set was dried the day of collection in Georgia; the other set was shipped to Peoria before drying. The latter set was shelled and dried as soon as possible after arrival in Peoria to prevent further toxin formation. Of the 57 samples dried in Georgia, total aflatoxin levels were less than 20 ng/g in 82%; the average total aflatoxin level in all samples was 36 ng/g. In the matching 57 samples dried in Peoria after shipment, total aflatoxin levels were below 20 ng/g in 70% of the samples; the average aflatoxin levels in all samples that had been dried later was 78 ng/g. These results indicated that aflatoxin did form during shipment of the 1979 freshly harvested corn from Georgia.

We are now reporting the results of a study on freshly harvested 1980 corn samples from Georgia in which we attempted to prevent mold growth and aflatoxin formation by use of Monoprop. Monoprop is a mixture of 1 part propionic acid plus 1 part versite. Versite, a horticultural vermiculite, serves as an inert support facilitating the handling of propionic acid in the field or laboratory.

METHOD

Sample Collection and Handling

Six 15-ft rows of 1980 mature dent corn were harvested from each of 67 fields in the coastal plain region of Georgia from July 22 to September 16, 1980. One set of samples consisted of every sixth ear of husked corn and was placed the day of collection in an oven at 60°C. Drying took 72 h, after which the sample was shipped to NRRC in Peoria to be shelled, prepared for analysis, and analyzed for aflatoxins. The re-

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maining husked ears of corn which had not been dried were placed in corrugated cardboard boxes packed with newspapers for shipment to NRRC. Samples were in transit an average of 7 days. The average temperature to be expected in the areas through which the samples were shipped and at the time of the year they were shipped was 74°F. In these areas over the years, the average daily maximum temperature was 84°F; average daily minimum temperature was 64°F. Upon arrival in Peoria, each undried ear corn sample was separated into 5 equivalent subsamples. One of the 5 subsamples was shelled and dried as soon as possible (overnight, 80–90°C). The other 4 subsamples were handled as follows: One was stored 3 weeks before shelling and drying; a second was stored 6 weeks. Two sets of samples were placed in airtight plastic bags with 5% Monoprop (5% of the weight of the ear corn or 2.5% propionic acid). One treated sample was stored 3 weeks, the other, 6 weeks. Storage was at 78–85°F.

Preparation of Subsamples for Analysis

Each sample of shelled corn was coarsely ground in a Straub disc mill. The coarsely ground corn was finely ground to pass a No. 20 sieve in a 6-in. Raymond hammer mill fitted with a screen with 1/8-in. perforations before being blended in a Hobart planetary mixer.

Aflatoxin Analysis

Portions of each subsample (50 g) were analyzed for aflatoxin by the method designated as the CB (Contaminants Branch) method, adopted by both the AOAC (3) and the American Association of Cereal Chemists (4).

Results and Discussion

The average number of ears in each of the subsamples was 16; the average weight of the shelled corn from each subsample was 1.5 kg.

Aflatoxin incidences and levels in the 6 sets of subsamples with varying storage times and Monoprop treatment are summarized in Table 1. The set of samples that was dried in Georgia before shipment obviously best represents aflatoxin occurrence in 1980 corn in that state. The fields had been sampled to be representative of the state. The incidence of samples with detectable aflatoxin was 93%; the incidence of samples with aflatoxin at levels equal to or greater than 20 ng/g (the Food and Drug Administration guideline) was 75% as contrasted with a 37% incidence of samples with detectable toxin in 1979 Georgia corn and an 18% incidence of samples of violative aflatoxin levels in the same year (2). The average total aflatoxin levels in all 1980 corn samples collected and dried in Georgia was 217 ng/g; in 1979; the average level was 36 ng/g.

In 1980, a comparison of the results of aflatoxin analysis of the subsamples dried in Georgia before shipment and of those obtained on subsamples dried in Peoria upon arrival revealed no overall differences in levels of aflatoxin determined or in the incidences of samples with detectable or violative levels of aflatoxins (Table 1). The samples were dried in Georgia the day of collection. The average time in transit was 7 days at an average temperature about 74°F. The average total aflatoxin level in all samples dried in Georgia was 217 ng/g; the average level in samples dried in Peoria immediately after arrival was 202 ng/g. All samples were husked as they were collected. The average toxin level in samples stored 3 weeks in Peoria before drying was 417 ng/g; the average level in samples stored 6 weeks before drying was 387 ng/g. There was a statistically significant increase in total aflatoxin levels when stored samples were compared to unstored samples.

A highly significant effect was associated with the Monoprop treatment, in that there were decreases in total aflatoxin levels when samples were stored in plastic bags with 5% Monoprop (2.5% propionic acid). In fact, levels were decreased by a factor of about one-half (Table 1). The concentration of Monoprop used may have been high enough to affect aflatoxin levels. There have not been reports of aflatoxin destruction in corn caused by propionic acid, but the hemiacetal, aflatoxin B_{2a}, does form slowly in acidic aqueous or acetone solutions (5, 6). Lower levels (0.4–1.0%) of propionic acid have been found to be effective in inhibiting growth in high moisture (e.g., 25%) shelled corn (7). We used higher concentrations (2.5%) of propionic acid because our samples were ears of corn and penetration might be more difficult.

The 1980 Georgia samples were separated into 3 groups according to moisture levels at harvest when the samples were collected: low (12.7–18.7%), medium (19.1–24.9%), and high (25.7–47.6%). The higher moisture samples initially had lower levels of total aflatoxin than the lower moisture samples (Table 2) but reached comparable aflatoxin levels during transit from Georgia to Peoria. The aflatoxin level of the samples with the higher moistures increased during transit by a factor of 2.66, which is significantly different from the expected ratio of 1.00 if there were no difference. In 1979 (2), the samples

Table 1. Comparisons of aflatoxin levels in 1980 corn samples dried in Georgia and dried in Peoria after shipping, after storage, or after treatment with 5% Monoprop [a]

			Shipped to NRRC		Stored 3 weeks before drying				Stored 6 weeks before drying			
	Dried in GA		before drying		Untreated		Treated		Untreated		Treated	
Total aflatoxin, ng/g	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%
ND	5	7.4	0	0	3	4.5	5	7.4	1	1.5	6	9.0
<20	12	17.9	18	26.9	13	19.4	12	17.9	7	10.4	6	9.0
20–99	28	41.8	21	31.3	17	25.4	27	40.3	20	29.8	29	43.3
100–499	16	23.9	22	32.8	19	28.4	20	29.8	24	35.8	21	31.3
500–999	2	3.0	3	4.5	7	10.4	2	3.0	7	10.4	3	4.5
≥1000	4	6.0	3	4.5	8	11.9	1	1.5	8	11.9	2	3.0
% incidence		93		100		96		93		99		91
% ≥20 ng/g		75		73		76		75		89		82
% ≥100 ng/g		33		42		51		34		58		38
Average level (ng/g), all samples		217		202		417		120		387		157
Average level (ng/g), positive samples		235		202		436		130		392		172

[a] Six sets of 67 matched samples.

Table 2. Geometric mean (ng/g) total aflatoxin for 1980 corn samples grouped by initial moisture at harvest

Moisture, %	No. of samples	No. of samples with increased aflatoxin	GD[a]	PD[b]	PD/GD
12.7–18.7	20	11	57.96	62.40	1.08[c]
19.1–24.9	22	13	86.95	72.88	0.84
25.7–47.6	25	16	26.16	69.55	2.66*

[a] GD = Dried in Georgia the day of harvest or collection.
[b] PD = Dried in Peoria after shipment from Georgia.
[c] Least significant ratio assuming 20 samples per mean is 2.18.
* Ratio significantly different from 1.00 at the 0.05 level.

with the highest moisture levels at harvest also had the lowest aflatoxin levels and changed most during transit from Georgia to Peoria.

In future studies, we plan to investigate the minimum levels of propionic acid required to prevent aflatoxin formation in corn after collection and the reaction of propionic acid on aflatoxin in corn.

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Presumptive Screening Test for Seminal Acid Phosphatase Using Sodium Thymolphthalein Monophosphate

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The use of sodium thymolphthalein monophosphate for presumptive seminal acid phosphatase testing is discussed. Recent evidence reveals that the dye (*o*-dianisidine) used in conjunction with α-naphthyl phosphate for seminal acid phosphatase testing is a carcinogenic hazard. Numerous tests for seminal acid phosphatase were conducted with swabs from sexual assault kits to compare both substrates. Results of tests using sodium thymolphthalein or α-naphthyl phosphate correlated exactly. Sodium thymolphthalein is an effective alternative for preliminary seminal fluid analysis because of its high degree of selectivity and stability, and because it eliminates the potential health hazard.

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The forensic scientist, when confronted with clothing or material involving sexual assault, requires a sensitive, quick screening test for seminal fluid. The screening test would indicate to the examiner that seminal fluid may be present and further analysis is required. The test would save considerable time locating individual seminal stains.

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Of the numerous components of seminal fluid, the detection of the enzyme acid phosphatase serves as a useful preliminary exam. Although not unique to seminal fluid, its abundance and persistence make it feasible to use as a presumptive test on material involving suspect seminal fluid. Numerous substrates for detection of acid phosphatase have been used by forensic serologists. The principal screening test for seminal acid phosphatase (SAP) uses α-naphthyl phosphate as a substrate and an azo-dye reagent (Fast Blue B) as a chromogen. Addition of these chemicals to suspect seminal stains results in the rapid formation of a brightly colored complex from colorless components. This convinces many analysts of the possible presence of human prostatic secretion (1)

Recent investigations have shown that the benzidine congeners (*o*-dianisidine) and benzidine-based dyes used in SAP reactions may pose a carcinogenic threat. Evidence indicates that humans as well as other animals can revert benzidine-based dye to benzidine by the enzyme azoreductase. This enzyme acts on numerous azo-compounds in vivo and in vitro (2).

Because of the large amount of SAP testing and the potential carcinogenic effect of the azo-dyes, this laboratory is currently using sodium thymolphthalein monophosphate as a substitute substrate for presumptive analysis. This reaction consists of acid phosphate hydrolysis of sodium thymolphthalein monophosphate to thymolphthalein and phosphate (3). Raising the pH terminates the hydrolysis while simultaneously developing an intense blue color. This substrate has a high degree of selectivity for prostatic enzyme and is less affected by nonprostatic acid phosphatase (4). To date, no known carcinogenic effects have been reported for sodium thymolphthalein (5).

Experimental

Reagents

(a) *Citrate buffer.*—Dissolve 29.4 g trisodium citrate dihydrate in 1 L water (Solution A), and add 2.1 g citric acid monohydrate to 100 mL water (Solution B). Add enough Solution B to about 900 mL Solution A to bring pH to 5.95 (25°C). This buffer is stable (4–8°C) ≥6 months (4).

(b) *Sodium thymolphthalein monophosphate.*—(Obtained from Sigma Chemical Co.). Dissolve 0.185 g sodium thymolphthalein in 100 mL buffer, to pH 6.0 (4).

(c) *Color developer.*—Prepare by adding 1 g sodium hydroxide to 5 mL water.

If less buffer is required for SAP testing, cut reagent concentrations proportionally. Use Brij-35 as wetting agent.

Examination

Ninety-seven tests were conducted on control, saliva, and vaginal swabs and clothing from rape evidence kits. The swabs and clothing containing suspect seminal stains were saturated with sodium thymolphthalein solution. After 5 min, 2 drops of color developer were added, and color intensity of the enzyme reaction was noted. A 5-min reaction time was minimum for good color development with moderate acid phosphatase levels, while not allowing time for low level substances such as vaginal fluids to develop much color, if any.

As a reference to each test, α-naphthyl acid phosphate and Fast Blue B salt were used and the color reactions were compared with those of thymolphthalein monophosphate. The reactions were graded as strong, medium, weak, and no reaction. A strong reaction yields a deep dark blue, while a medium reaction is blue. Those judged weak gave a greenish light-blue tint, and no reaction gave no color. Other material tested included bovine, potato, and wheat germ acid phosphatases. Standard saliva, vaginal, and seminal fluid were also examined.

Results

The strong, medium, and weak reactions of thymolphthalein correlated exactly with the reference purple reactions of Fast Blue B on 96 examinations. In one instance in which the vaginal swab was bloody, the thymolphthalein reagent gave a weak reaction and the Fast Blue B reagent gave a negative reaction. However, it has been reported in the literature that blood may interfere with the α-naphthyl phosphate reaction and cause an inexperienced person to incorrectly judge the reaction positive (6). We found that it is easier to judge the blue thymolphthalein reaction in the presence of blood rather than the purple reaction of Fast Blue B and α-naphthyl phosphate.

Standard saliva and vaginal swabs were negative. Bovine acid phosphatase and potato acid phosphatase were also negative; however, large amounts of wheat germ acid phosphatase gave a greenish-blue tint. Some concentrated vaginal and buccal stains gave a similar color. In light of this, any substance giving a greenish-blue tint would be deemed negative; only dark blue reactions would be accepted as presumptive evi-

dence for seminal acid phosphatase. A blank swab gave a yellowish reaction.

Summary

Sodium thymolphthalein monophosphate serves as an effective substitute for presumptive seminal acid phosphatase testing. It possesses a high degree of selectivity and reagent stability, and eliminates the potential health hazard from the benzidine direct and congener dyes.

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Rapid Extraction and Gas-Liquid Chromatographic Determination of Benzoic and Sorbic Acids in Beverages

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A rapid method for extraction and quantitative determination of sorbic and benzoic acids in carbonated drinks and fruit juices is described. Acidified sample aliquots are transferred onto an Extrelut column. Acid preservatives are then eluted from the column with a mixture of ethyl ether-petroleum ether. Content of preservatives in the concentrated ethereal extract is readily determined by temperature-programmed gas-liquid chromatography without the need to prepare derivatives.

Benzoic and sorbic acids are widely used as preservatives in several kinds of food. Gas-liquid chromatographic (GLC) methods are quick and sensitive and have frequently been used for determination of these compounds. Two methods are generally used for isolating these preservatives from food: steam distillation followed by extraction of the distillate by ether (1–3) or by a mixture of organic solvents (4, 5) and direct extraction of the acidified sample by an organic solvent (6–11). More recently, Schindler and Spröer (12) developed a method for the extraction of benzoic acid and the esters of *p*-hydroxybenzoic acid from enzymatic preparations, which uses an Extrelut prepacked column containing silica with a large pore granular structure. It is used for extracting lipophilic substances from the aqueous phase by liquid-liquid partition chromatography.

The present work demonstrates that good results can be obtained by using the extraction technique on the Extrelut column, followed by gas-liquid chromatography, for the quantitative determination of benzoic and sorbic acids in soft drinks and fruit juices.

METHOD

Reagents and Apparatus

(a) *Extrelut® column.*—E. Merck, Darmstadt, GFR.

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(b) *Gas-liquid chromatograph.*—Perkin-Elmer Model 900 equipped with flame ionization detector. Conditions: 6 ft × 1/8 in. id stainless steel column containing 10% SE-30 on 80–100 mesh Chromosorb W-HP; column temperature programmed from 50 to 170°C at 24°/min; injection port and detector 230°C; flow rate (nitrogen) 42 mL/min.

(c) *Internal standard solution.*—0.3 g *n*-butyl benzoate in 100 mL methanol.

Preparation of Standard Curve

Prepare 5 mL standard solutions containing 1, 2, 4, 6, 8, 10, 12, and 13 mg benzoic and sorbic acids/mL, respectively, in solutions of internal standard. Analyze 2 μL aliquot by GLC. Standard curve was selected such that highest possible correlation coefficient was obtained: r^2 = 0.9986 and 0.9960 for benzoic acid and sorbic acid, respectively.

Experimental Procedure

Acidify 25 mL portion of sample (fruit juice or previously decarbonated soft drink) to pH = 1 with 8 drops of concentrated HCl and pipet 20 mL directly onto Extrelut column. Wait 15 min and then rinse column with ≤2 mL water. Elute preservatives from column with 60 mL mixture of ethyl ether–petroleum ether (3 + 1). Evaporate eluate to dryness under stream of dry air at room temperature. Pipet 2 mL internal standard solution onto dry residue and subject 2 μL aliquot of this final solution to GLC analysis under specified conditions. Calculate concentrations of organic acids relative to internal standard from measurement of peak areas by triangulation. Concentration of preservative is given by the following equation:

$$\text{Preservative (ppm)} = 2000\,C/A$$

where C = concentration of preservative on the standard curve in mg/mL; A = quantity of sample taken for analysis in mL; 2000 = dilution and unit transformation factor.

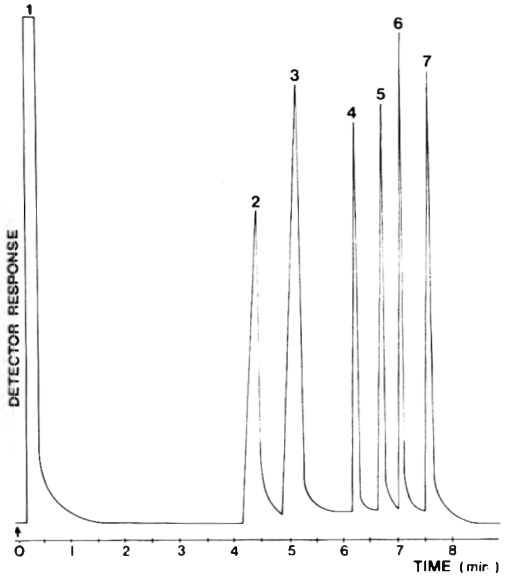

Figure 1. Representative chromatogram of mixture of preservatives. 1, solvent; 2, sorbic acid; 3, benzoic acid; 4, butyl benzoate (internal standard); 5, methyl *p*-hydroxybenzoate; 6, ethyl *p*-hydroxybenzoate; 7, propyl *p*-hydroxybenzoate.

Results and Discussion

GLC analysis is performed in approximately 6 min and peaks are well resolved. Figure 1 is a typical chromatogram showing the separation of benzoic and sorbic acids under the specified conditions.

Interference from other fruit juice or soft drink constituents in the chromatogram was not observed. An attempt was made to determine the acids on the SE-30 column operating isother-

Table 1. Recoveries of sorbic and benzoic acids in beverages

	Sorbic acid			Benzoic acid		
Sample	Added[a] mg	Found[b] mg	Rec.,[c] %	Added[a] mg	Found[b] mg	Rec.,[c] %
Soft drinks	9.76	9.4 ± 0.2	95.9 ± 1.9	10.16	9.7 ± 0.2	95.4 ± 1.5
	21.14	20.4 ± 0.3	96.4 ± 1.6	22.02	21.2 ± 0.5	96.2 ± 2.4
Fruit juices	9.76	8.8 ± 0.2	89.9 ± 2.0	10.16	9.0 ± 0.1	89.1 ± 2.5
	21.14	18.8 ± 0.5	88.9 ± 2.4	22.02	19.5 ± 0.4	88.6 ± 1.8

[a] In 20 mL beverage.
[b] Standard deviations for 9 determinations.
[c] Standard deviation for % recovery was determined from % recovery for each of 9 determinations.

mally (2, 5), but tailing of the solvent peak sometimes interfered in measurement of sorbic acid, as was observed by Clarke et al. (2). Such interference did not occur with temperature programming. Peaks were Gaussian with a slight tailing which does not interfere with the measurements, and no variation of the standard curves with time was observed. The fact that these conditions are also adequate for the analysis of the esters of *p*-hydroxybenzoic acid is shown by Figure 1.

Recovery experiments were based on a total of 9 determinations carried out on soft drinks and fruit juices to which known amounts of sorbic and benzoic acids were added.

The range of initial concentration used in this study was 500 to 1000 ppm. The results obtained are shown in Table 1. Recovery was satisfactory for soft drinks. The lower recoveries in the extraction of fruit juices may be due to the retention of preservatives in particles of fruits suspended in the juice and retained in the filter on the top of the column. The present method was fast (total time for analysis is about 30 min), relatively inexpensive, and sensitive for routine determinations of benzoic and sorbic acids in soft drinks, although the recovery from fruit juices is slightly less than optimum.

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NEW PUBLICATIONS

Dictionary of Food Ingredients. By Robert S. Igoe. Published by Van Nostrand Reinhold, 135 W 50th St, New York, NY 10020, 1982. 192 pp. Price: $17.95.

Information on 996 permissible food ingredients approved by the U.S. Food and Drug Administration is provided by this reference book. An alphabetical listing supplies descriptions of each ingredient's function, properties, and applications. Typical usage levels are given and chemical formulations for many of the ingredients. Included is such information as solubility, pH, synonyms, and compatibility with other ingredients. The ingredients are grouped by function into 14 categories such as antioxidants, sweeteners, preservatives, and vitamins. The author describes the general functional characteristics and applications of each category.

Advances in Steroid Analysis. Analytical Chemistry Symposia Series Volume 10. Proceedings of the Symposium on the Analysis of Steroids, Eger, Hungary, May 20–22, 1981. Edited by S. Görög. Published by Elsevier Scientific Publishing Co., PO Box 211, 1000 AE Amsterdam, The Netherlands, 1982. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017. 464 pp. Price: US $104.75/Dfl. 225.00. ISBN 0-444-99711-3.

The 71 papers which comprise this volume form a state-of-the-art survey of the two main fields of steroid analysis—biological-clinical and industrial-pharmaceutical. Hormones, sterols, D vitamins, bile acids, cardiac glycosides, and the methodological problems involved in their analysis are covered. Papers in the biological-clinical field deal mostly with the application of protein binding (mainly immunological) methods, with chromatographic separation and gas chromatographic-mass spectroscopic methods included. Methods used in the industrial-pharmaceutical field are mostly thin-layer chromatography and high performance liquid chromatography. Modernized versions of the classical methods of spectrophotometry and polarography are also reported.

Handbook of Organic Analytical Reagents. Edited by K. L. Cheng. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1982. 544 pp. Prepub. price: $74.00, outside U.S. $84.00. ISBN 0-8493-0771-6.

This work-bench reference describes classical and modern organic analytical reagents used in the analysis of inorganic substances, organic and biochemical substances, and in instrumental analyses. Theoretical discussions of applications of organic analytical reagents with respect to masking and demasking, specificity, sensitivity, solvent extractions, and other chemical separations are presented. The following information is given on each reagent: general method of synthesis, purity criteria and method of purification, physical properties, molecular formula, water of crystallization, structural formula, absorption spectral data. Special data are given for chelating, precipitating, and redox reagents. References and ideas for future research are included.

Methodology of Analytical Toxicology. Volume II. Edited by Irving Sunshine. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1982. 272 pp. Prepub. price: $42.50, outside U.S. $49.00. ISBN 0-8493-0771-1.

Volume II is a continuation of a series of alternative systematic analyses of medication suspected to be the cause of illness.

Preservation of Food by Ionizing Radiation. Edited by Edward S. Josephson and Martin S. Peterson. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1982. Volume I: 384 pp., prepub. price $99.50, outside U.S. $110.00 ISBN 0-8493-5323-8; Volume II: 352 pp., prepub. price $92.50, outside U.S. $106.00, ISBN 0-8493-5324-6; Volume III: 320 pp., prepub. price $92.00, outside U.S. $104.00, ISBN 0-8493-5325-4.

The underlying science and technology of this food preservation process, ionizing radiation, is described in detail from the chemical, physical, and biological effects on

food to the problems and limitations encountered, and the successful application to specific food groups. Discussions are included on radiation dosimetry, health physics for people working with the irradiation process, adjusting radiation doses for specific needs, lethal effect on microorganisms and insects, and effects of irradiation and relationships to changes in the flavor, texture, and color of food.

Chemical Analysis of Inorganic Constituents of Water. Edited by Jon C. Van Loon. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1982. 264 pp. Price $74.50, outside U.S. $85.00. ISBN 0-8493-5209-6.

This volume describes the analysis of chemicals in water from maintaining sample integrity to analytical procedures, through the interpretation of results. Determination of various chemicals in a sample are described via the following analytical methods: EDTA complexometric titration, atomic absorption spectrophotometry, atomic emission spectrometry, ion-selective electrode methods, ion chromatography, and flame photometry. Procedures, reagents, equipment, and calculations are included for each method presented. The analytical potential of recently developed methods is also discussed.

Recommended Health-Based Limits in Occupational Exposure to Pesticides. Report of a WHO Study Group. World Health Organization Technical Report Series No. 677. 1982. Available from WHO Publications Centre USA, 49 Sheridan Ave, Albany, NY 12210. 110 pp. Price: Sw.fr. 8.–. Arabic, French, and Spanish editions in preparation. ISBN 92-4-1206772.

WHO has embarked on a program of establishing internationally recommended health-based occupational exposure limits. Recommendations are being made by a series of study groups, each convened to evaluate a group of related substances.

The present report deals with the pesticides malathion, carbaryl, lindane, and dinitro-*o*-cresol, all widely used in agriculture or public health or both. Primary emphasis is laid on epidemiological studies of industrial and agricultural workers. Particular attention is paid to relevant recommendations of other international bodies. Each section of the report contains recommendations for further studies on the health effects of the 4 pesticides and the report concludes with some general recommendations for pesticide research.

Chemistry & Biochemistry of Marine Food Products. Edited by Roy E. Martin and George J. Flick. Published by the AVI Publishing Co., PO Box 831, Westport, CT 06881, 1982. 475 pp. Price: U.S. $49.50, other countries $54.50. ISBN 0-87055-408-5.

A new reference book, this represents the first attempt to draw together in one forum the present knowledge concerning the chemistry and biochemistry of marine food products. It includes large amounts of data and references translated from foreign sources now appearing for the first time in U.S. scientific literature. It includes information on seafood technology, byproduct utilization, and recent advances in marine product preservation.

Statistical Methods for Food and Agriculture. By F. E. Bender, L. W. Douglass, and A. Kramer. Published by AVI Publishing Co., PO Box 831, Westport, CT 06881, 1982. 345 pp. Price: U.S. $25.00, other countries $27.50. ISBN 0-87055-391-7.

Theory, uses, and applications of statistics as decision-making tools in the food and agriculture industries are described in this book. Designed as a self-contained study unit for use as a textbook in colleges and universities, it presents statistical techniques used in research analysis and business operations.

BOOK REVIEW

Environmental Carcinogens. Selected Methods of Analysis. Volume 4—Some Aromatic Amines and Azo Dyes in the General and Industrial Environment. Edited by H. Egan, L. Fishbein, M. Castegnaro, I. K. O'Neill, and K. Bartsch. Published by International Agency for Research on Cancer, Lyon Cedex 2, France, 1981. IARC Publication No. 40. Available from WHO Publication Centre, 49 Sheridan Ave, Albany, NY 12210. xiii + 350 pp. Price $30.00. ISBN 92-8-3211405.

Originally considered to be a potential hazard only to workers in the dye, rubber, and chemical industries, exposure to carcinogenic aromatic amines and related compounds is now a more general environmental problem. This book, Volume 4 in a series dedicated to the analysis of environmental carcinogens, provides reviews and analytical monographs contributed by various authors.

By virtue of the number of identified compounds which are potentially carcinogenic to man, the list of monographs is not complete. The monographs, which have all been published elsewhere, are consistent in their style and format and provide adequate details on the methodologies, hazards, and applicabilities of each technique. The vast majority of the techniques described utilize GC or HPLC, reflecting the important contribution made by advances in the chromatographic sciences to research in environmental toxicology.

The literature dealing with the analysis of aromatic amines and related compounds is well reviewed in a chapter by Fishbein. Most of the book is devoted to those compounds found in the industrial environment. Their occurrence is reviewed in Chapter 4. A similar review of the scope of the problem in the non-industrial environment is not included, presumably because the occurrence and long term applications of potentially carcinogenic amines found in food, clothing, drugs, and tobacco smoke is not well defined, whereas the discussion in this book is restricted to specific examples.

This book is recommended as an up-to-date source of reference of interest primarily to those engaged in the analysis of aromatic amines. Informative and well referenced review chapters on the carcinogenicity, metabolic activation, and occurrence of these substances also provide a more general appeal.

C. M. RILEY

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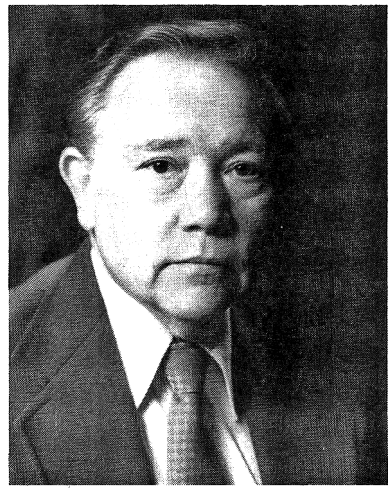
FOR YOUR INFORMATION

A New Year and a New President for AOAC—Warren Bontoyan of EPA



Warren R. Bontoyan, a chemist and Chief of the Environmental Protection Agency's Chemical and Biological Investigations Branch with laboratories located at Beltsville, MD, is President of AOAC for 1983. He is active in the development and standardization of chemical methods of analysis and in national and international scientific organizations. He is listed in *Who's Who in America.*

The Environmental Protection Agency Beltsville Laboratories which he directs are concerned with pesticide residues, pesticide degradation, and toxic impurities and/or contaminants associated with pesticides in technical and commercial products. He and his colleagues have given and continue to give valuable assistance to many state, municipal, and foreign government laboratories. Mr. Bontoyan has 25 publications on the analysis of pesticides, related chemicals, and toxic impurities and has given numerous presentations at national and international meetings on special studies and analysis of pesticides. He is the editor of the *Environmental Protection Agency Manual of Chemical Methods for Pesticides and Devices* published by the AOAC.

Warren Bontoyan has been an active member of the AOAC since 1960. Since 1962, he has served as Associate Referee for a number of topics, was General Referee for Pesticide Formulations from 1974 to 1981, and was made a Fellow of the AOAC in 1973.

He has served on the Long Range Planning Committee, Gas Chromatography Committee, International Cooperation Committee, Committee A, Quality Assurance Committee, Symposia and Special Programs Committee, Centennial Committee, as AOAC representative to the Collaborative International Pesticides Analytical Council (CIPAC), and as a member of the Board of Directors.

Mr. Bontoyan is the U.S. Member of CIPAC, a member of the World Health Organisation (WHO) Panel of Experts on the Specification and Chemistry of Pesticides, the American Association of Pesticide Control Officials (AAPCO), the Food and Agriculture Organisation (FAO) Panel of Experts on Pesticide Specifications, American Chemical Society (ACS), Alpha Chi Sigma, International Union of Pure and Applied Chemistry (IUPAC), and the American Institute of Chemists, and is a Fellow of the American Institute of Chemists. He organized and chaired a session on the Safety Aspects of Formulated Products at the Fifth International Congress of Pesticide Chemistry (IUPAC) held in Japan in 1982.

He has 27 years of federal service distributed among the U.S. Department of Agriculture, the U.S. Food and Drug Administration, and the U.S. Environmental Protection Agency, and is a graduate of the University of Maryland.

Warren Bontoyan lives in Perry Hall, Maryland, is married to the former Gladys Francis Daughaday and has two children, Warren and Suzanne. He has been active in the Boy Scouts of America and is a church council member of St. Michael's Lutheran Church.

AOAC Supporters—Near and Far

Industry here and abroad is aware of the need to support an independent methods validation association. Leatherhead Food R.A., Surrey, England, Agway, Inc., Syracuse, NY, Chevron Chemical Co., Richmond, CA, Kellogg Co., Battle Creek, MI, Thomas J. Lipton, Inc., Englewood Cliffs, NJ, Quaker

Oats Co., Chicago, IL, and ITT Continental Baking Company, Inc., Rye, NY, are the most recent additions to the list of those indicating this awareness by providing financial support to AOAC.

Meetings

February 13–17, 1983: International Conference on Oils, Fats, & Waxes; University of Auckland, Auckland, New Zealand. Contact: S. G. Brooker, International Conference on Oils and Fats, Chemistry Dept, University of Auckland, Private Bag, Auckland, NZ.

February 15–19, 1983: American Academy of Forensic Sciences 35th Annual Meeting, Stouffer's Cincinnati Towers & Convention Center, Cincinnati, OH. Contact: AAFS, 225 S Academy Blvd, Suite 201, Colorado Springs CO 80910; 303/596-6006.

March 1–3, 1983: Symposium on Soil Erosion and Crop Productivity, Executive Tower Inn, Denver, CO. Contact: ASA, CSSA, SSSA Headquarters Office, Attn: Denver Symposium, 677 South Segoe Rd, Madison, WI 53711.

April 7–8, 1983: Committee E-11 on Statistical Methods, ASTM Headquarters, Philadelphia, PA. Contact: Bill Hulse, 215/299-5507.

April 19–21, 1983: AOAC 8th Annual Spring Workshop and Exposition, Sheraton West Hotel, Indianapolis, IN. Contact: Lawrence Sullivan, Indiana State Board of Health, 1330 West Michigan St, Indianapolis, IN 46206; 317/633-0224.

June 5–8, 1983: 66th Canadian Chemical Conference, Convention Centre, Calgary, Alberta, Canada. Contact: Arvi Rauk, MCIC, Department of Chemistry, Univ. 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 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.

July 17–23, 1983: SAC 83—International Conference and Exhibition on Analytical Chemistry, University of Edinburgh, Edinburgh, Scotland. Contact: P. E. Hutchinson, Secretary, Analytical Div., Royal Society of Chemistry, Burlington House, London, W1V OBN, UK.

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.

Course Offered

X-ray spectrometry and X-ray powder diffraction.—The Physics Dept of the State University of New York at Albany is offering their annual 2-week summer courses in X-ray spectrometry, June 6–13, 1983, and in X-ray diffraction, June 20 to July 1, 1983. Neither course requires previous knowledge or experience. Course time will be divided equally between lecture and laboratory-problem solving. First week of both courses will cover basic principles, techniques, and practical applications.

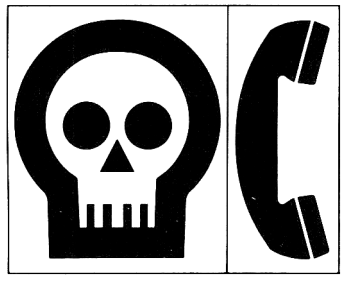
For the spectrometry course, the 2nd week will continue with fundamentals and practical applications with emphasis on advanced principles and techniques, absorption-enhancement corrections including mathematical methods, computer calculations, and computer automation of modern X-ray spectrometers. Both wavelength dispersive and energy dispersive methods will be used.

For the powder diffraction course, 1st week emphasis will be on camera and film techniques, X-ray instrumentation, especially the diffractometer and its use, identification of powder patterns, multi-phase identification, and fundamentals of quantitative analysis. Second week will emphasize diffractometer alignment, complex quantitative analysis, complex powder identifications, computer automation of diffractometers, and computer search-match methods.

The registration fee for each course is $800.00 for one week or $1500 for a 2-week session. For further information and to register, contact Prof. Henry Chessin, SUNYA, Dept. of Physics, 1400 Washington Ave., Albany, NY 12222. Phone 518/457-8339.

Poison Control Movement Announces New Symbol in Public Awareness Campaign

This distinctive symbol used to identify poison control centers and their activities soon will become familiar to health care professionals. The logo has been adopted by the American Association of Poison Control Centers for use by its more than 400 members nationwide. It is hoped that the general public and the medical and hospital community will learn to associate this symbol with a place to call for immediate help—their local poison control center. For information, call Ken Clair, 202/452-1188.



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