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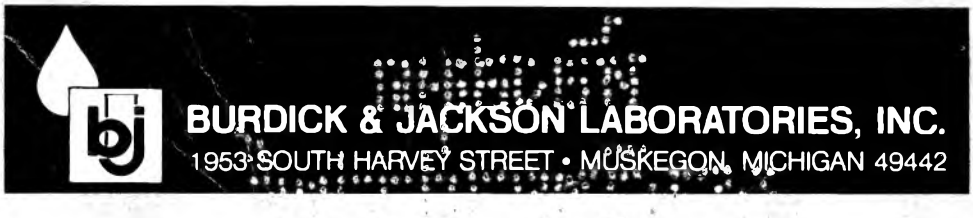

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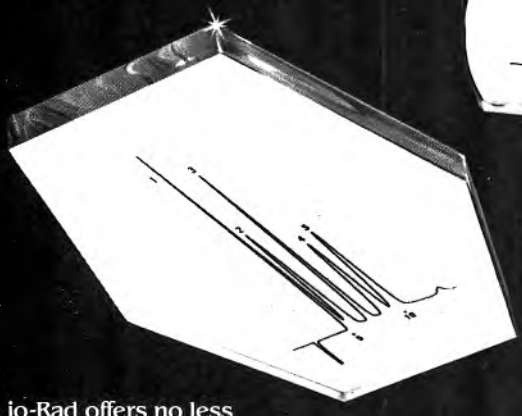

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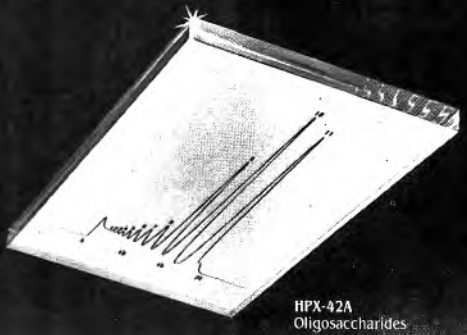

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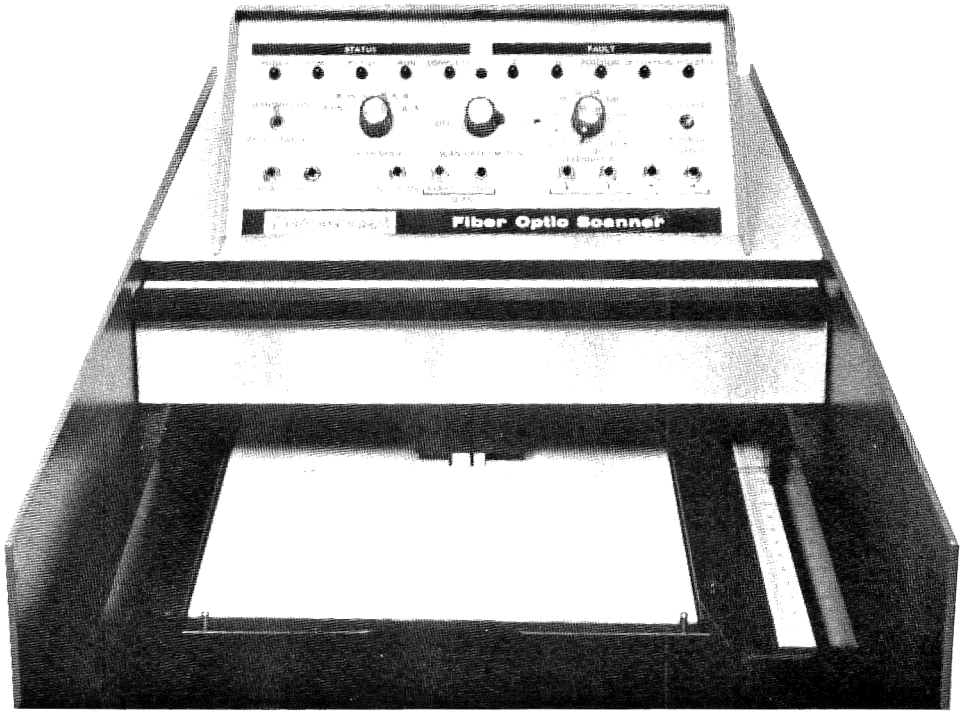

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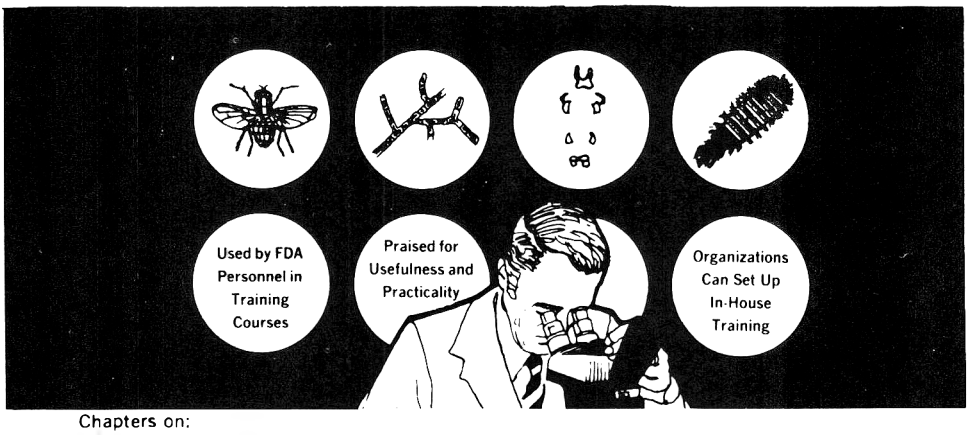

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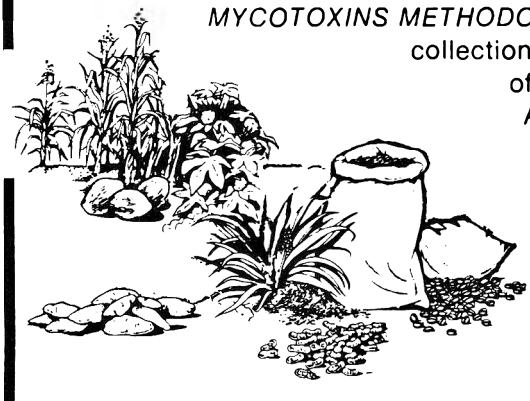

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Modified Colorimetric Method for Determining Indole in Shrimp

WAI LUN CHEUK and GUNNAR FINNE
Texas A&M University, Animal Science Department, Seafood Technology Section, College Station, TX 77843

A modified spectrophotometric method for measuring indole in shrimp is described. The method was developed to eliminate the time-consuming steam distillation and expensive instrumentation required by the official AOAC methods. Indole is extracted with light petroleum from trichloroacetic acid-precipitated shrimp muscle. The extracted indole, soluble in light petroleum, is reacted and re-extracted with Ehrlich's reagent; indole in the form of a rose indole complex can be determined spectrophotometrically. When shrimp at various degrees of decomposition were analyzed for indole by the modified method as well as by the official AOAC colorimetric method, the correlation coefficient between the data from the 2 methods was 0.98.

Among numerous methods suggested as chemical quality indexes of fresh and frozen shrimp, the determination of indole has recently attracted much attention. Even though indole was studied as an index of shrimp quality as early as 1946 (1), it was only recently that the Food and Drug Administration (FDA) proposed the use of indole levels as an indicator of decomposition in imported shrimp. In support of organoleptic tests, indole has already been used as a decomposition indicator in the seizure of frozen salad shrimp (2).

It is generally believed that indole is present in shrimp as a result of bacterial activity before freezing (3). The numerical levels for indole tolerance which correspond to organoleptic tolerance levels of decomposition have been established.

Traditionally, indole in seafoods has been determined by a colorimetric method (4) which involves a time-consuming steam distillation with subsequent cumbersome extraction of the distillate. More recent methods include gas-liquid chromatography (5), fluorometric analysis (6), as well as liquid chromatography (7). The latter 3 methods require well trained personnel and sophisticated and expensive instrumentation, and therefore are not suitable as quality control methods in the seafood industry.

When researching the mechanism of indole formation in shrimp, we developed a modified spectrophotometric method which is a rapid, simple, and convenient alternative to the official steam distillation colorimetric procedure. This modified method, which is based on the work of Happold and Hoyle (8), eliminates the steam distillation and shortens the analysis time from approximately 4 to 1 h per sample. Although the basic spectrophotometric principles are the same for both the official and the modified method reported here, this modified method takes advantage of using immiscible indole solvents for separation and purification rather than steam distillation. This simplified method could become a part of the quality control procedures in the seafood processing industry.

METHODS

Apparatus

(a) *Spectrophotometer.*—Perkin-Elmer, Model 124, double beam grating spectrophotometer with automatic recorder. Place slit selector at 0.5 nm, which, according to operation manual, provides bandpass of 0.5 nm.

(b) *Centrifuge.*—Damon/IEC refrigerated centrifuge Model B-20A.

Reagents

(a) *Trichloroacetic acid (TCA).*—Accurately weigh 6 g TCA (MCB Manufacturing Chemists, Inc., Norwood, OH) in 100 mL distilled water.

(b) *Light petroleum.*—Petroleum ether (Fisher Scientific, Fair Lawn, NJ) bp 37.1–57.5°C.

(c) *Ehrlich's reagent.*—Dissolve 9 g ACS grade *p*-dimethylaminobenzaldehyde (PABA) (MCB Manufacturing Chemists, Inc.) in 45 mL concentrated HCl in 250 mL volumetric flask and dilute to volume with ethanol.

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Table 1. Comparison of AOAC colorimetric and modified colorimetric methods for determining indole in shrimp (μg indole/100 g shrimp, hydrated basis) decomposed under different conditions [a]

Sample	Temp.	Time	AOAC	Modified
1	22°C	13 h	5.85	5.82
2	22°C	16 h	53.79	62.50
3	22°C	18 h	120.45	103.90
4	ice	6 days	1.17	1.25
5	ice	9 days	4.21	5.00
6	ice	10 days	7.02	6.25
7	ice	11 days	9.12	10.00
8	ice	13 days	17.54	15.00
9	11°C	24 h	4.68	9.85
10	11°C	60 h	18.71	23.16

[a] Correlation coefficient = 0.98.

(d) *Standard indole solutions.*—Accurately prepare stock solution of 10 mg indole (Eastman Chemical Co., Rochester, NY) in 100 mL light petroleum. Use 1:10 dilution as working solution. Refrigerate indole solutions.

Shrimp Samples

Brown shrimp (*Penaeus aztecus*), obtained directly from shrimp trawlers in Aransas Pass, Texas, were immediately packed in ice and shipped to the laboratory in College Station. To induce different degrees of decomposition, shrimp were stored at 3 temperatures for various time intervals as shown in Table 1. After being sampled, shrimp were frozen and stored at −25°C until analysis (maximum of 5–6 days for repeated analysis).

Procedure

Homogenize 40 g shrimp with 80 mL TCA solution in a Waring blender 1 min. Add 80 mL ice-cold light petroleum and blend 1 min. Transfer homogenate to 250 mL centrifuge bottle and centrifuge 10 min at 10 000 rpm. Filter supernate through Whatman No. 1 paper under slight suction. Transfer filtrate to 250 mL separatory funnel. After the 2 layers have separated, transfer acid layer (lower) to second 250 mL separatory funnel.

Wash TCA-denatured protein precipitate separated by centrifugation with 40 mL light petroleum and filter as described above. Transfer filtrate to second 250 mL separatory funnel already containing TCA layer from first extraction. Shake vigorously 1 min and let 2 layers separate. Transfer lower acid layer to third separatory funnel and extract for third time with 40 mL light petroleum.

Combine all light petroleum extracts into 1 separatory funnel and extract indole with exactly 5 mL freshly prepared Ehrlich's reagent by vigorously shaking 1 min. The rose indole complex formed is insoluble in light petroleum and indole is thus quantitatively transferred to Ehrlich's reagent layer. When layers have separated and cleared, transfer part of lower colored layer to 1 cm path cell and read at 570 nm against reagent blank solution. If rose indole solution is not clear, centrifuge at low speed before reading in spectrophotometer. Determine indole concentration from standard curve. Rose indole complex from indole standard and from TCA-extracted shrimp is stable ≥4 h, and ordinary laboratory illumination does not affect its intensity.

Prepare standard curves as follows: For pure indole, accurately measure volumes from 0.5 to 4 mL (5 to 40 μg) stock indole solution into 80 mL TCA in separatory funnel. Re-extract indole by procedures described above and construct standard curve.

For shrimp spiked with indole, to 40 g peeled and deveined shrimp free of indole, add from 0.5 to 4 mL stock indole solution. Mix well and let all light petroleum evaporate. Extract indole from samples by procedure described above and construct standard curve.

Results and Discussion

Figure 1 shows the relationship between pure indole extracted from TCA and indole extracted from spiked shrimp. As indicated, indole extracted from spiked shrimp was recovered at a level of 94%. This recovery was uniform, independent of species and indole level. To incorporate the recovery factor into the extraction procedure, the standard curve used throughout this study was constructed from spiked shrimp free of indole. However, there is no reason why pure indole solutions cannot be used for construction of a standard curve when a recovery factor is used in the final calculations.

With either of the above procedures, the standard curve was linear in the region from 0 to 1.5 scale units (Figure 1). Assuming a reading confidence of 0.05 absorbance, the range of indole levels from 40 g sample would be from 5 to 125 μg/100 g shrimp. However, by increasing the sample size, a low level (1 μg/100 g shrimp) is detectable.

Shrimp at various degrees of decomposition (Table 1) were rapidly thawed inside water-tight plastic bags under running cold water. While still semifrozen, the shrimp were divided into 2 portions and analyzed immediately. One por-

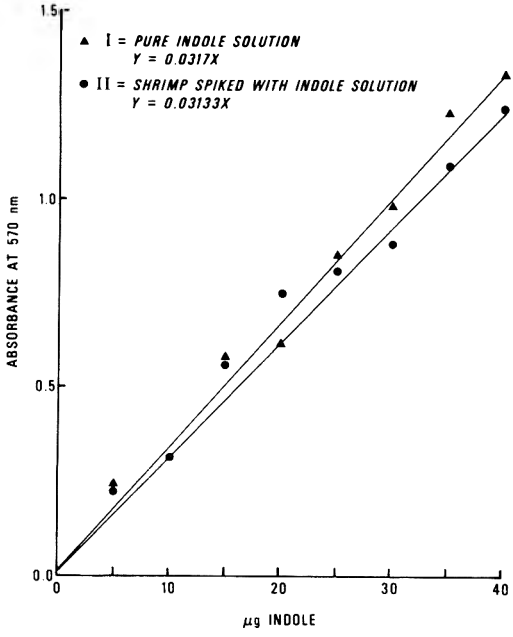

Figure 1. Standard curves for pure indole solution (I) and for shrimp spiked with indole (II).

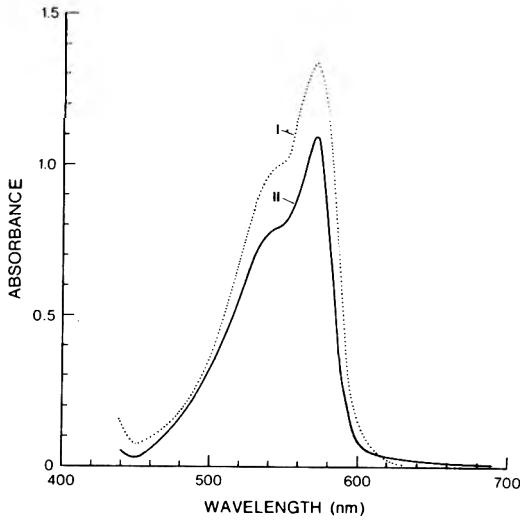

Figure 2. Visible absorption spectra of pure indole derivative and indole derivative extracted from decomposed shrimp: I, spectrum from decomposed shrimp; II, spectrum from pure indole.

tion was analyzed for indole according to the procedure described above, while the other was analyzed for indole according to the official AOAC steam distillation method. The data obtained for 10 different samples are shown in Table 1. As is evident from the table, there was close agreement between indole levels determined by the 2 methods. In the range from 1.0 to 120 μg indole/100 g shrimp, a correlation coefficient of 0.98 indicates good agreement between the 2 methods.

To compare the properties of the colored complex formed from pure indole to the complex extracted from decomposing shrimp, the 2 complexes were scanned in the visible spectral region. As is evident from Figure 2, the 2 complexes had similar visible absorption spectra, indicating that the extracted compounds from spoiled shrimp are indole-related substances.

In the official AOAC spectrophotometric method, steam-distilled and extracted indole reacts with *p*-dimethylaminobenzaldehyde to give a reddish compound. The reacting aldehyde, known as Ehrlich's aldehyde, is the principal ingredient in Ehrlich and Kovac reagents, used widely by microbiologists for detecting indole-producing bacteria in tryptone medium. The modified colorimetric method described here also used Ehrlich's aldehyde for color development. However, instead of separating indole from interfering compounds by the laborious steam-distillation procedure, this method uses a combination of time- and equipment-saving extractions. When a light petroleum solution of indole is extracted with Ehrlich's solution, the indole in the light petroleum fraction reacts with the aldehyde to form the colored complex. This complex, insoluble in light petroleum, is quantitatively transferred to the acidic Ehrlich's reagent.

This study has shown that indole can be extracted from decomposing shrimp by using light petroleum on TCA-precipitated shrimp muscle. The method has the advantages over comparable official methods of saving time and using less equipment. It is hoped that the method may be suitable as a much needed quality control tool for the seafood industry.

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EXTRANEOUS MATERIALS

Distinguishing Common Food-Contaminating Bat Hairs from Certain Feather Barbules

ALAN R. OLSEN
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Differences are described between common food-contaminating vespertilionid bat hairs (Chiroptera: Vespertilionidae) and similar-appearing downy feather barbules of passeriform birds, including cuticular scales, pigment distribution, and basal characteristics. Fifteen imported food samples analyzed over a 1½ year period were contaminated with these bat hairs.

The mission of the U.S. Food and Drug Administration (FDA) includes protecting the American consumer against the contamination of food by commensal animals. Recently, the FDA Los Angeles laboratory found an unexpectedly high number of imported food samples contaminated with bat hairs (Table 1). The presence of these hairs in a food indicates that the food is defiled and was handled under unsanitary conditions, probably in a structure where bats roost. Since no specific methodology exists to detect bat manure (guano) or other bat contaminants in food, the detection of bat filth rests on the ability of the analyst to recognize bat hairs.

The most common of these hairs are from bats of the cosmopolitan family Vespertilionidae, which include little brown bats (*Myotis*), pipistrelles, and others. These hairs are similar in appearance to the downy feather barbules of passeriform birds (Passeriformes), a group which includes the house sparrow among its many species. Since some members of both these groups readily invade and inhabit buildings, giving them the opportunity to despoil food supplies, the similarity of their respective hairs and barbules could be confusing to food sanitation analysts. Our informed conclusion is that misidentification of bat hairs as feather barbules occurs frequently.

Microscopically, vespertilionid bat hairs and passeriform downy barbules both look like slender (20–30 μm diameter) filaments with a striking pattern of black (pigmented) and white (unpigmented) bands. It is impossible to reliably distinguish the two with a widefield microscope. For this study, specimens were mounted in glycerin jelly (1) and compared at 400× magnification. The specimens were obtained both from food samples and from identified bat or bird pelts.

Food-Contaminating Bat Hairs

The cuticular scales of bat hairs are the coronal type. Hausman (2) likens these coronal scales to "a pile of tall tumblers placed one within the other, the upper rims representing the free ectal edges of the scales." Each coronal scale completely surrounds the hair shaft (Figure 1). Food-contaminating bat hairs have hastate coronal scales (Figure 2), which are a distinctive feature of most vespertilionid bat hairs (3). Unequal hastate coronal scales may give the hairs a dissymmetrical appearance (Figure 3).

Vespertilionid bat hairs lack medullae, and the centers are uniformly unpigmented. The pigment of these hairs is located in the cuticular scales (Figure 4), a condition which can be confirmed microscopically by lowering the microscope focal plane through the hair specimen and noting color changes. This condition can also be demonstrated by stripping scales away from the shaft (Figure 5). The base of a bat hair, like all mammalian hairs, narrows in diameter before ending in a bulbous root (Figure 6).

In addition to Vespertilionidae, hairs were examined from the major bat families Emballonuridae, Megadermatidae, Molossidae, Noctilionidae, Nycteridae, Phyllostomatidae, and Pteropidae. These other hairs bore little resemblance to vespertilionid hairs and passeriform barbules.

Passeriform Downy Feather Barbules

Downy barbules are found on the downy portions of covert feathers (4). They are the most resilient type of barbule, hence the most likely to survive food processing. These barbules lack cuticular scales and consist of a shaft or pennu-

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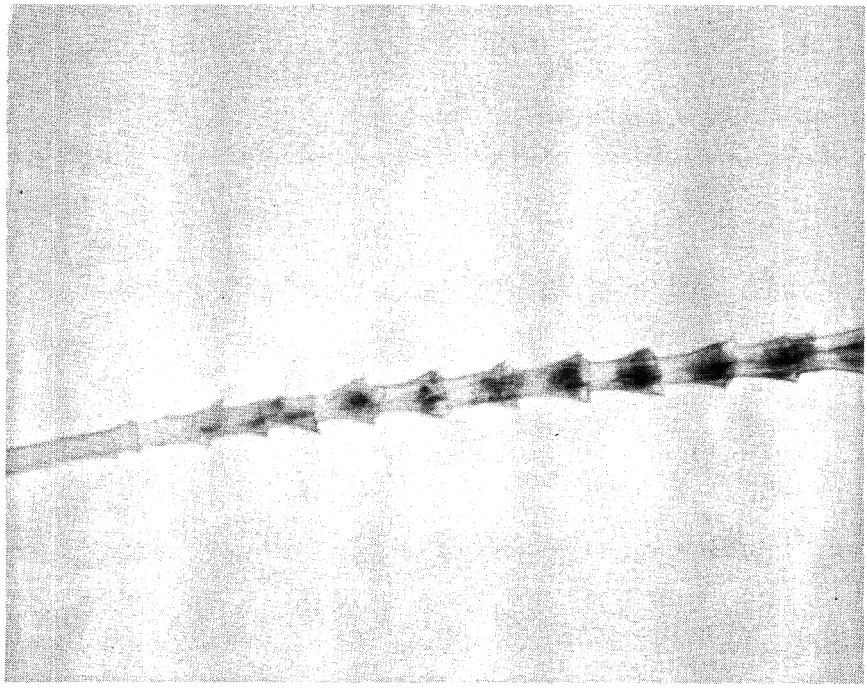

Figure 1. Pipistrelle bat hair with distinct coronal scales (400×).

lum of rather uniform composition with swellings or nodes at regular intervals (Figure 7). A barbule does not narrow basally, and the basal end is flattened where it is attached to the ramus.

The pigment of passeriform downy barbules is located inside the node. The outer surface of the node and the entire internode are unpigmented (Figure 8). This is the opposite of the vespertilionid hairs in which the pigment is external and the central core is unpigmented.

Other kinds of birds whose downy barbules exhibit color patterns similar to passeriform barbules are the gulls and waders (Charadriiformes), woodpeckers (Piciformes), some owls (Strigiformes), and some parrots (Psittaciformes). The barbules of these birds (including Passeriformes) can be identified only from specimens that are intact from base to tip, an uncommon occurrence in processed foods. Passeriform

Table 1. Summary of imported food samples containing bat hair contaminants found by FDA Los Angeles laboratory from 1/79 to 6/80

Type of food	No. of samples	Country of origin
Bean curd	1	China
Chutney	1	India
Fungus, dry black	6	China, Hong Kong, Taiwan
Fungus, dry white	1	Taiwan
Ginger, pickled	1	Hong Kong[a]
Lasagne	1	Italy
Plums, dry salted	2	Hong Kong, Taiwan[b]
Tamarind	2	Mexico[b]

[a] Also contained a bat bug (Hemiptera:Polyctenidae) carcass.

[b] Also contained passeriform downy feather barbules.

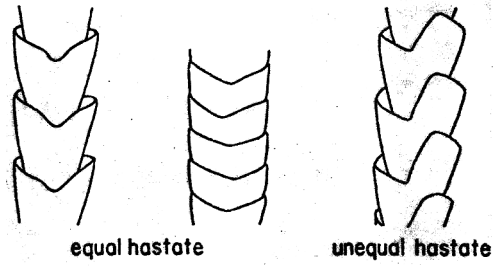

Figure 2. Types of hastate coronal scales (from Benedict).

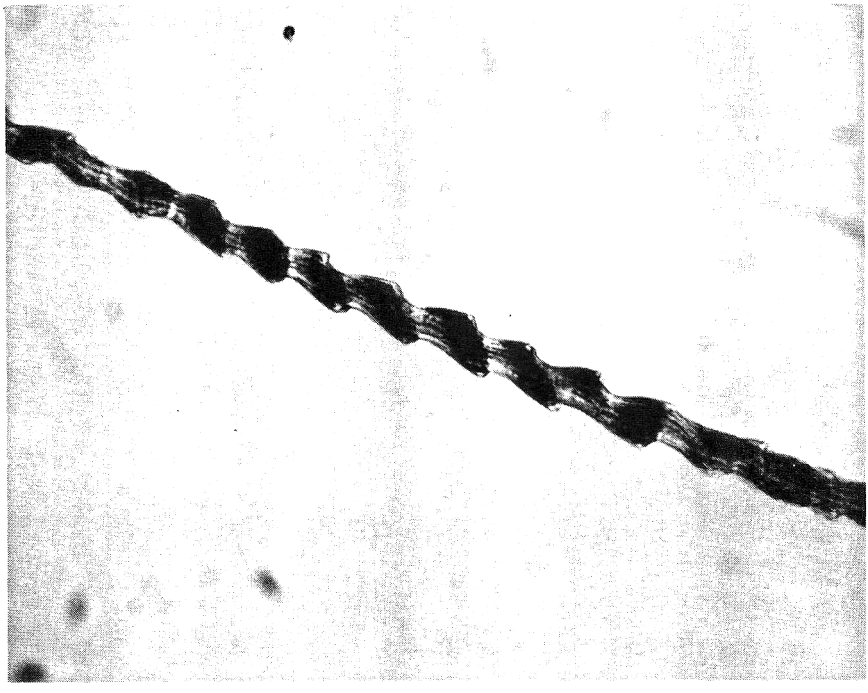

Figure 3. Little brown bat (*Myotis*) hair. Note dissymmetrical appearance caused by unequal hastate scales (400×).

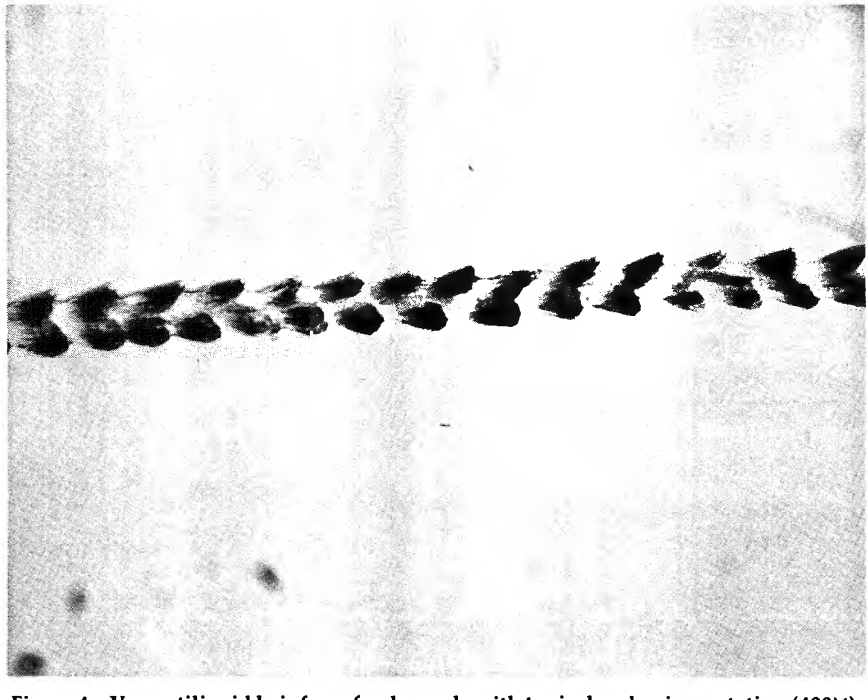

Figure 4. Vespertilionid hair from food sample with typical scale pigmentation (400×).

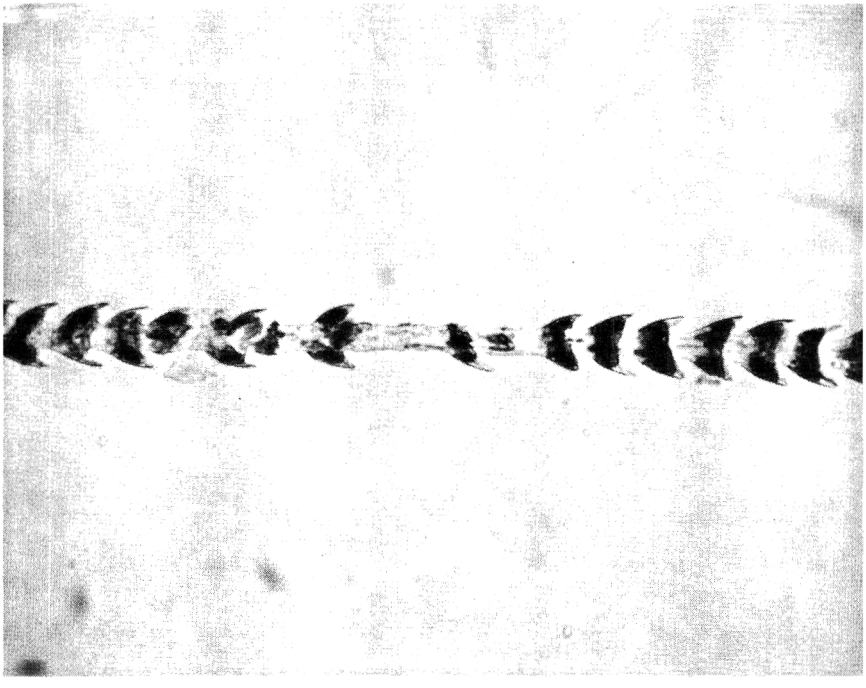

Figure 5. Bat hair with scales stripped to show unpigmented central shaft (400×).

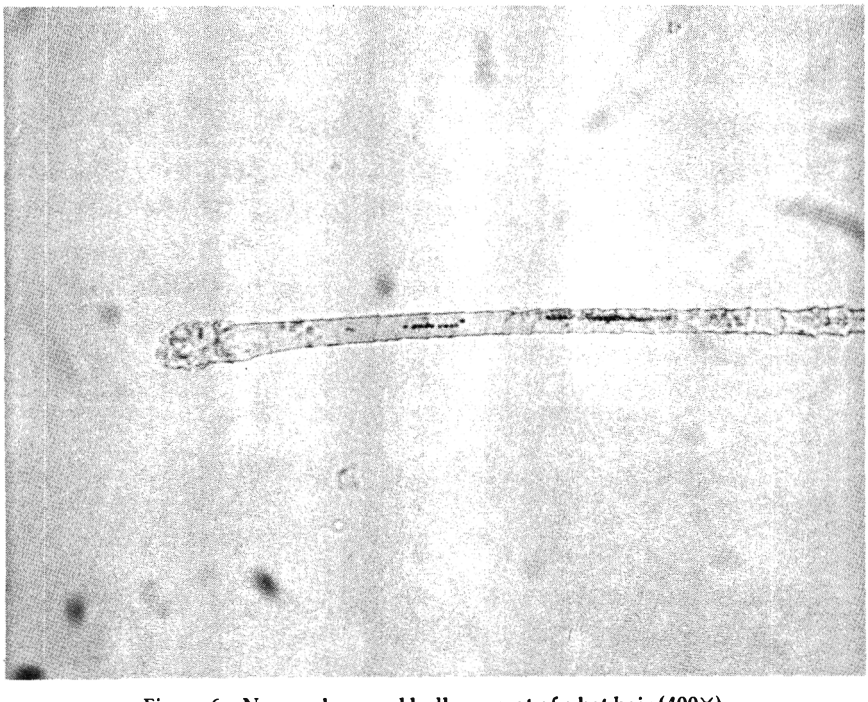

Figure 6. Narrow base and bulbous root of a bat hair (400×).

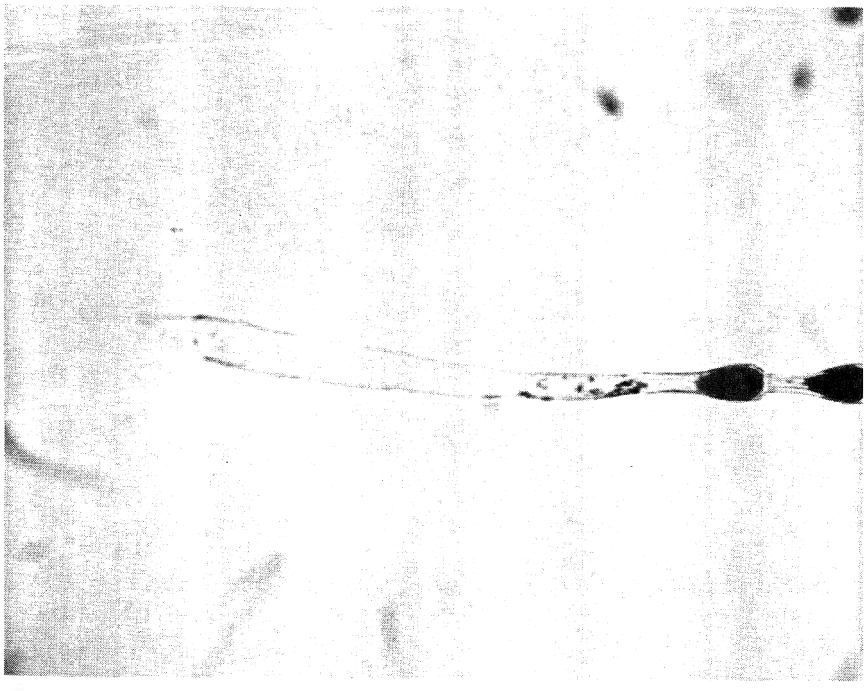

Figure 7. Passeriform downy barbule. Note the lack of scales, flattened base, and uniform pennulum diameter (400×).

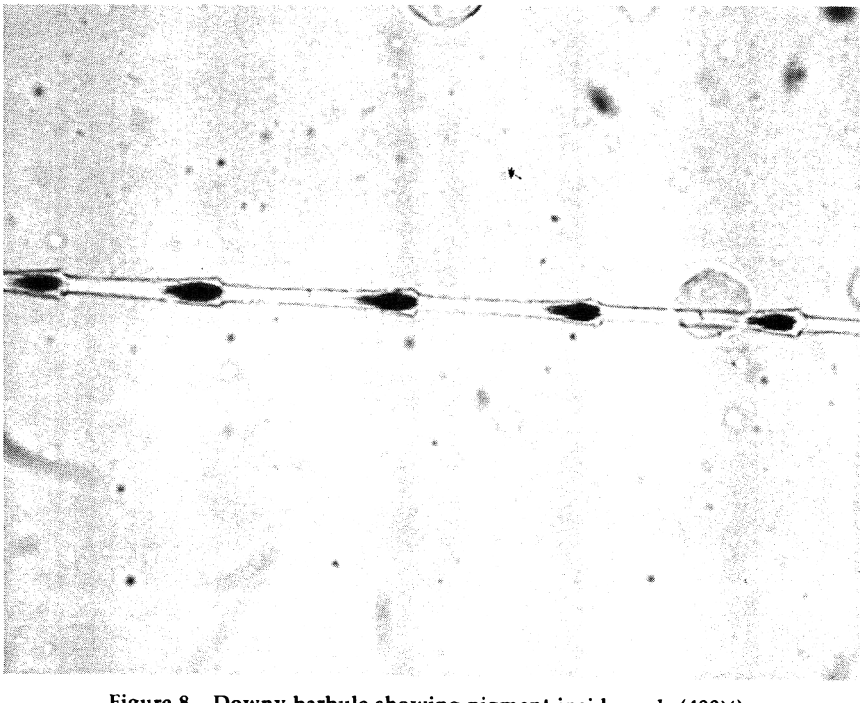

Figure 8. Downy barbule showing pigment inside node (400×).

birds were used for comparative purposes since they are the most likely contributors of this type of food contaminant.

Acknowledgment

The author thanks Donald Patton and the Los Angeles Museum of Natural History for assistance in obtaining authentic bat hairs.

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AOAC

ALCOHOLIC BEVERAGES

Rapid Check on Proof of Rums

FABIÁN ALVAREZ-NAZARIO
Treasury Department of Puerto Rico, Bureau of Alcoholic Beverages Taxes, San Juan, Puerto Rico 00905

The apparent proof of undistilled rum is determined by alcohol hydrometer and by refractometer, and correlated with the true proof and solids content.

A convenient method of determining the true proof of distilled spirits containing small amounts of dissolved solids is the obscuration method. A direct determination of solids is made for each sample, and 0.4 degree proof is added to the hydrometer apparent proof for each 100 mg solids per 100 mL sample.

Results can be checked by a determination of the apparent proof of the sample, using a refractometer and corresponding tables. True proof is calculated on the basis of the following equations:

$$TP = HP + sF_1$$

$$TP = RP - sF_2,$$

where TP = true proof of the sample, HP = apparent proof obtained using an alcohol hydrometer, RP = apparent proof obtained using a refractometer, s = solids content of sample, and F_1 and F_2 = constant factors.
From the above equations,

$$s = (RP - HP)/(F_1 + F_2)$$

$$TP = HP + (RP - HP)F_1/(F_1 + F_2).$$

Thus, in a series of samples in which HP, RP, and s are determined directly and the true proof (TP) is determined by the obscuration method, we may expect a constancy in the relationships:

$$(RP - HP)/s = (F_1 + F_2)$$

$$(TP - HP)/(RP - HP) = F_1/(F_1 + F_2).$$

That such is the case can be seen from the last 2 columns of Table 1, the results obtained with typical Puerto Rican rums. The calculated true proofs and solids contents obtained when using the average factors 0.0125 and 0.32 are shown in Table 2.

Factor F_1 is the obscuration factor of 0.004 degrees proof per mg/mL, which is valid for spirits containing less than 600 mg solids per mL and in the alcohol range of 80–100°P.

The effect of a given amount of solids on the hydrometer and refractometer readings depends on the alcohol concentration and on the nature of the solids. For spirits containing more than 600 mg solids/100 mL, the nature of the solids may be different and their determination by drying is more uncertain. In such cases it is

Table 1. Experimental data on Puerto Rican rums and calculated factors

Sample	HP[a]	RP[b]	Solids[c]	TP[d]	$(F_1 + F_2)$	$F_1/(F_1 + F_2)$
1	79.38	80.90	112	79.83	0.0136	0.30
2	79.32	80.78	121	79.80	0.0121	0.33
3	79.52	81.08	124	80.02	0.0126	0.32
4	79.80	81.84	173	80.49	0.0118	0.34
5	79.31	82.10	217	80.18	0.0129	0.31
6	78.65	82.28	289	79.81	0.0125	0.32
7	78.73	82.56	305	79.95	0.0126	0.32
8	78.55	82.27	308	79.78	0.0121	0.33
9	78.30	82.70	344	79.68	0.0128	0.31
10	78.92	83.61	379	80.44	0.0124	0.32
11	78.18	84.10	480	80.10	0.0123	0.32
Mean					0.0125	0.32
SD					0.0005	0.01

[a] HP = Apparent proof by hydrometer.
[b] RP = Apparent proof by refractometer.
[c] Solids in mg/100 mL.
[d] TP = True proof by obscuration method.

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Table 2. Calculated proof and solid content

Sample	Calcd proof	Diff.[a]	Calcd solids[b]	Diff.[a]
1	79.87	+0.04	122	+10
2	79.79	−0.01	117	−4
3	80.02	0.00	125	+1
4	80.45	−0.04	163	−10
5	80.20	+0.02	223	+6
6	79.81	0.00	290	+1
7	79.96	+0.01	306	+1
8	79.74	−0.04	298	−10
9	79.71	+0.03	352	+8
10	80.42	−0.02	375	−4
11	80.07	−0.03	474	−6

[a] Calculated value minus true value.
[b] Solids in mg/100 mL.

necessary to determine the true proof on a distillate of the sample in order to use the relationship

$$F_1/(F_1 + F_2) = (TP - HP)/(RP - HP).$$

Procedures for determining hydrometer apparent proofs are described in the *Gauging Manual* (1) and in official AOAC methods. The refractometers commonly used in the alcoholic beverages industry are the immersion type, and readings can be converted to % alcohol by volume by means of refractometric tables in the AOAC *Official Methods of Analysis* (2). Other types of refractometers may be used if observation temperatures lie between 17.5 and 25°C, the range covered by the tables. Alcohol hydrometers can be substituted by suitable pycnometers which determine both the apparent and the distillation proof of the sample by using the AOAC tables. The tables give % alcohol volume from specific gravity determinations.

The simultaneous determination of alcohol and solid content by density and refractive index measurement in the undistilled sample has been used for beer, wine, whisky, and brandy for more than 100 years (3, 4). The novelty of the present method, as devised for rums containing less than 600 mg solids/100 mL, is that it does not require density and refractive index determinations at a specified temperature, but is based on routine procedures of apparent proof determinations at room conditions, using alcohol hydrometers and refractometers.

Received October 2, 1980. Accepted January 23, 1981.

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DRUG RESIDUES IN ANIMAL TISSUES

Gas-Liquid Chromatographic Determination of Sulfamethazine in Swine and Cattle Tissues

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A gas-liquid chromatographic (GLC) method is described for determining sulfonamide residues in animal tissues, with specificity for 7 sulfonamides. Residues are extracted from tissues with acetone-chloroform, fatty substances are removed, and the sulfonamide residue is methylated with diazomethane in acetone-ether to render it amenable to determination by gas-liquid chromatography on an all-glass column suitable for direct on-column injection and a Ni electron-capture detector. Quantitation is achieved by external standardization. The method has a validated limit of sensitivity of 0.10 ppm with the corresponding control values for all tissues being less than 0.01 ppm. Satisfactory recoveries have been obtained for sulfamethazine in swine and cattle tissues. Specificity for sulfamethazine in the presence of sulfathiazole, sulfaquinoxaline, sulfadimethoxine, sulfabromomethazine, sulfaethoxypyridazine, and sulfachloropyrazine is attained by resolution of the respective methyl derivatives on the GLC column.

The historical development of sulfonamides over almost 50 years, beginning with the synthesis of sulfanilamide, is described by Linkenheimer and Stolzenberg (1). They also describe the pharmacologic characteristics of 4 sulfonamides, including sulfamethazine, in swine. More recently, others have reported on the pharmacokinetics of sulfonamides in various food-producing animals, i.e., Koritz et al. (2) and Bevill et al. (3) report on sulfathiazole in sheep and Bourne et al. (4) report on sulfamethazine in lambs.

In metabolism studies, Nielsen (5) described the metabolic pattern of 4 sulfonamides, including sulfamethazine, in cows, and Bevill et al. (6) described the metabolism of sulfamethazine in lambs. In both studies, several metabolites, including the N^4-acetyl derivative, were found in urine samples, following single intravenous injections. No metabolite identification in tissues was attempted, presumably because these were not radiotracer studies.

The largest percentage of the sulfonamide market consists of sulfamethazine used in combination with antibiotics as feed supplements to promote growth and prevent disease in swine and cattle. Much of the violative residue levels found by the U.S. Department of Agriculture in swine liver have been attributed to sulfamethazine, having been determined essentially by the method of Tischler et al. (7), with minor modifications. Their method uses the Bratton-Marshall colorimetric reaction with *N*-(1-naphthyl)-ethylenediamine dihydrochloride as the chromogenic reagent. It was originally developed to determine therapeutic blood levels where the drug under study was known. The reagent forms a colored product with any diazotized aromatic amine and hence will not distinguish individual sulfonamides. The method also generates false positives in swine liver if the liver is not held at dry-ice temperature before analysis. This method is satisfactory for assaying tissues from animals having received a known treatment, particularly at higher residue levels as used by Mutha et al. (8) to determine the biological half-life of sulfamethazine in calf tissues. However, it was considered desirable to develop a single, sensitive, specific method which was not susceptible to false positive values in swine liver, the target tissue for swine.

The gas-liquid chromatographic (GLC) procedure described here has been proven applicable for determining residues of sulfamethazine, sulfathiazole, sulfaquinoxaline, sulfadimethoxine, sulfabromomethazine, sulfaethoxypyridazine, and sulfachloropyrazine. The procedure uses the solvent extraction system of the Tischler method for sulfa residues with a higher ratio of solvent volume to sample size. The retention times for the respective methylated derivatives of these 7 sulfonamides are different on each of 2 GLC columns, hence this method can be used to quantitate the sulfa residue level and confirm the identity of the particular sulfa present. If further confirmation of identity is required, these extracts are directly amenable to GLC–mass spectrometric (MS) analysis.

The GLC procedure is very similar to that of Goodspeed et al. (9), developed for confirmation studies, except that we deleted the derivatization with pentafluoropropionic anhydride. That step

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did somewhat increase the response but, in our laboratories, it resulted in a high, variable gas chromatographic baseline and somewhat low recovery (50–60%) for sulfamethazine. These baseline problems were assumed to be due to the presence of multicomponents in the tissue extract which reacted with the fluoro reagent; Daun (10) did not encounter this problem in feeds fortified at the 25–50 ppm level with 4 sulfonamides.

METHOD

Reagents and Apparatus

Rinse all clean glassware thoroughly with methanol and let dry before using. All solvents should be distilled in glass, suitable for pesticide analyses, as supplied by Burdick and Jackson Laboratories, Inc., or equivalent.

(a) *Sulfamethazine standard solution.*—Prepare stock solutions containing 1, 2, and 10 μg sulfamethazine/mL acetone.

(b) *Derivatizing reagent.*—Prepare diazomethane by ethereal basic ethanol distillation of 21.5 g *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald, Aldrich Chemical Co., Milwaukee, WI 53233) with 200 mL ether as described in their Diazald kit, Cat. No. Z10, 025-0. (*Note:* Read Diazald kit instructions carefully for safe handling of diazomethane. For further information on handling diazomethane, see ref. 11.) After distillation of the second portion (40 mL) of ether, transfer ether solution of diazomethane through funnel to narrow-mouth bottle and cap tightly with a polyseal cap. When stored in the freezer compartment of a refrigerator, this solution retains its efficiency as a methylating agent for at least 1 month.

(c) *Gas chromatograph.*—Tracor Model 550, or equivalent, with ^{63}Ni electron-capture detector, suitable for on-column injections and fitted with a 6 ft × 2 mm id glass column packed with 10% OV-101 on 60–80 mesh Gas-Chrom Q or 5% OV-7 on 100–120 mesh Gas-Chrom Q (Applied Science Division, Milton Roy Co. Laboratory Group, PO Box 440, State College, PA 16801). Operating conditions: injector 290°C, column oven 280°C (for 5% OV-7 column) or injector 270°C, column oven 260°C (for 10% OV-101 column); detector 285°C; argon–methane (95 + 5) carrier gas at 30 mL/min; detector purge flow rate same as carrier flow; retention time for N^1-methyl derivative of sulfamethazine ca 5 min.

Procedure

Pulverize sufficient dry ice in bowl of Hobart food chopper to chill bowl and grater thoroughly. Attach 9 in. vegetable slicer attachment with $^3/_{32}$ in. shredder plate and chop tissue to a fine particle size. Continue adding sample and chopping until complete sample is chopped. If necessary add more dry ice to maintain sample in frozen state during chopping procedure. Store sample in a freezer (−20°F) until dry ice has dissipated.

Transfer representative 15 g sample of the tissue to a Waring blender jar and blend 5 min with 150 mL acetone–chloroform (1 + 1). Filter by gravity through glass-fiber paper. Transfer 100 mL aliquot to 250 mL round-bottom flask with 24/40 joint and add 10 mL 1N aqueous HCl. Evaporate organic solvents on a rotary evaporator with flask submerged in 35–40°C water bath. (In the case of muscle and fat, some extracted fat will prevent complete removal of solvents.) Add 50 mL *n*-hexane to the 1N HCl phase and quantitatively transfer both phases to 125 mL separatory funnel. Rinse evaporation flask with additional 5 mL 1N HCl and transfer rinse to 125 mL separatory funnel. Shake contents of separatory funnel gently by inverting funnel and returning to upright position once a second for 30 s. Draw off lower phase (1N HCl) into test tube. (Centrifugation may be required to avoid transfer of emulsified solvent which tends to bump during hydrolysis step.)

Repeat extraction of hexane phase with fresh 5 mL portion of 1N HCl, and after phase separation combine it with first portion in test tube. Submerge test tube in boiling water bath 15 min. (This procedure hydrolyzes the N^4-acetyl derivative if present.) To determine only residues of parent sulfonamide, omit this hydrolysis step. Cool to room temperature if hydrolysis step is used.

In the case of fat samples, extract 1N HCl solution with 15 mL methylene chloride to remove excess residual fat globules and discard methylene chloride phase. Buffer HCl by adding 25 mL saturated trisodium citrate; then adjust pH with pH meter to 5.55–5.65 by adding ca 2.5 mL aqueous 3N sodium hydroxide. Transfer buffered solution to 125 mL separatory funnel and extract with four 15 mL portions of methylene chloride by shaking vigorously 30 s with each portion. Recheck pH of aqueous phase after first extraction and re-adjust to 5.55–5.65 if necessary. Collect the 4 methylene chloride extracts in 100 mL evaporation flask and evaporate solvent on rotary film evaporator. Dissolve residue in 1 mL acetone. Add 1 mL diazomethane solution and

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Table 1. Recovery of sulfamethazine from swine tissues

Tissue	Fort. level, added, ppm	Av. % rec. (No. of replicates)	Range, %
Liver	0.10	95 (4)	80–109
	0.20	90.5 (3)	88.5–92
	0.50	83.6 (2)	82.1–85.1
	1.00	93.7 (1)	
Overall average (std dev.)		91.1 (8.54)	
Kidney	0.10	89.8 (2)	80.5–99.0
	0.20	91.3 (2)	90.0–92.5
	0.50	94.9 (2)	90.4–99.4
	1.00	86.6 (1)	
Overall average (std dev.)		91.2 (6.7)	
Muscle	0.10	103.5 (2)	90–117
	0.20	97.5 (2)	91–104
	0.50	100.5 (2)	97.3–103.8
	1.00	100.9 (1)	
Overall average (std dev.)		100.6 (9.9)	
Fat	0.10	92.0 (2)	91–93
	0.20	97.8 (2)	97–98.5
	0.50	92.6 (2)	91.7–93.5
	1.00	94.5 (1)	
Overall average (std dev.)		94.2 (2.7)	

let mixture stand 15 min with intermittent swirling. Evaporate solvent with gentle stream of air in hood and dissolve residue in acetone. Inject 2–5 μL aliquots onto the chromatographic column and measure sulfonamide content against external sulfonamide standard derivatized (methylated) at same time as sample.

Results and Discussion

In the original development of the method, an acid hydrolysis step was used because previous work by Linkenheimer and Stalzenberg (1) had shown appreciable quantities of hydrolyzable metabolite(s), presumably either *N*-acetyl or *N*-conjugated metabolites, present in swine urine but no more than traces, if any, in swine blood. Hence, an acid hydrolysis step was included and a recovery study with sulfamethazine and its N^4-acetyl derivative was run in liver. Tissues were fortified by dipping the appropriate amount of N^4-acetyl derivative or sulfamethazine in 1.0 mL acetone onto the thawed tissue, allowing the acetone to evaporate completely at room temperature, and then extracting the sample as described under *Procedure*. Six recoveries from each tissue were run for the N^4-acetyl derivative and 8 recoveries from each tissue were run for sulfamethazine, using the acid hydrolysis step. The average recovery and standard deviation for N^4-acetyl derivative in the 4 tissues (24 assays) was 94.9% with a standard deviation of 11.9%. Average recoveries and standard deviations for sulfamethazine for the 8 assays each of liver, kidney, muscle, and fat were 84.3 ± 11.0%, 87.5 ± 10.9%, 99.6 ± 10.7% and 86.8 ± 10.5%, respectively. Recovery values of sulfamethazine from swine and cattle tissue obtained from laboratory fortification studies run without the acid hydrolysis step (recommended procedure) are reported in Tables 1 and 2, respectively. Typical chromatograms for liver fortified at 0.1 ppm and control liver processed through the procedure are shown in Figure 1. A typical chromatogram showing the resolution of the methyl derivatives of 7 sulfonamides is shown in Figure 2.

Twenty Yorkshire × Hampshire hogs were given feed medicated with AUREO SP 250 premix which furnished 100 g sulfamethazine, 100 g chlortetracycline, and 50 g penicillin per ton of feed from weaning to market weight (200 lb). Four groups of 5 animals each were sacrificed at 0, 7, 10, and 14 days after withdrawal from medicated feed. Liver and kidney samples taken from these animals were assayed by both the GLC (with hydrolysis) procedure and the Bratton-Marshall procedure reported by Tischler et al. (7) with the following modifications: 5 mL concentrated hydrochloric acid was added to the initial combined sample extracts (chloroform–acetone) before evaporation of the solvents at 50°C, and the resultant oily residue was trans-

Table 2. Recovery of sulfamethazine from cattle tissues

Tissue	Fort. level, added, ppm	Av. % rec. (No. of replicates)	Range, %
Liver	0.10	96.9 (3)	90–103
	0.20	110.8 (2)	107–114.5
	0.50	104.8 (2)	100.3–109.0
	1.00	99.8 (1)	
	2.00	98.8 (1)	
Overall average (std dev.)		102.2 (7.2)	
Kidney	0.10	96.8 (3)	92–104
	0.20	92.3 (2)	90–95.5
	0.50	96.6 (2)	95.4–97.8
	1.00	86.6 (1)	
	2.00	96.9 (1)	
Overall average (std dev.)		94.6 (4.9)	
Muscle	0.10	100.7 (3)	84–124
	0.20	89.5 (2)	83.5–95.5
	0.50	100.2 (2)	99.6–100.9
	1.00	95.1 (1)	
	2.00	98.5 (1)	
Overall average (std dev.)		97.2 (11.8)	
Fat	0.10	99.6 (3)	94–107
	0.20	90.5 (2)	87–94
	0.50	85.8 (2)	80.3–91.2
	1.00	88.5 (1)	
	2.00	79.1 (1)	
Overall average (std dev.)		91.0 (8.7)	

ferred to a separatory funnel containing 10 mL distilled water with six 25 mL portions of hexane and two 3 mL portions of acetone. The chloroform partitioning step at the alkaline pH was performed as described under "Milk Penicillin G and Other Aromatic Amines Present" on p. 52 of ref. 7. The average recovery of sulfamethazine from liver was 79.7% (range 64–95%).

The residue values obtained by both methods are shown in Table 3. The comparable values obtained by the 2 methods agree well after the 0-day samples, showing that the 7, 10, and 14 day samples contain no acid-hydrolyzable metabolites. These residue values agree very closely with the total radioactive residues and with the rate of tissue residue disappearance found in a ^{14}C-sulfamethazine swine metabolism study (J. Rosen, Rutgers University, New Brunswick, NJ, private communication). In that study, the total radioactive residues in liver were: 4 ppm at 3 h withdrawal, 0.9 ppm at 2 days, 0.1 ppm at 5 days, 0.03 ppm at 10 days, and 0.01 ppm at 15 days. We were unable to ascertain the reason for the higher values obtained by GLC on the 0-day samples.

To verify that sulfamethazine reacts with diazomethane to form the N^1-methyl derivative as reported by Roder and Stuthe (12) and to use the authentic compound to determine the percent conversion of sulfamethazine to sulfanilamide, N^1-(4,6-dimethyl-2-pyrimidinyl)-N^1-methyl, at residue levels, this authentic compound was prepared by the method of Asplin et al. (13). The crude reaction product was crystallized 4 times from 95% ethanol and had a melting point of 214°C (range 213.5–214°C). Its C-13 NMR spectrum was consistent with the structure of sulfanilamide, N^1-(4,6-dimethyl-2-pyrimidinyl)-N^1-methyl, and indicated a mole purity of 90–95%. Only one UV-absorbing spot (less polar than sulfamethazine) was observed in each of 2 thin layer chromatographic solvent systems vs silica gel and only one peak was observed on each of 2 GLC columns, which was at the same retention time as that of the reaction product of sulfamethazine and diazomethane. The chemical ionization mass spectra using methane as the reagent gas were identical for this compound prepared by the 2 routes with an (M + H) ion at m/e 293 and major fragment ions at m/e 166 and 138. The conversion of sulfamethazine over the range of 1–10 μg/mL to the N^1-methyl derivative by the reaction with diazomethane was reproducible with a yield of approximately 90% compared to that of the authentic compound.

Limited (6–8 runs on each) recovery studies

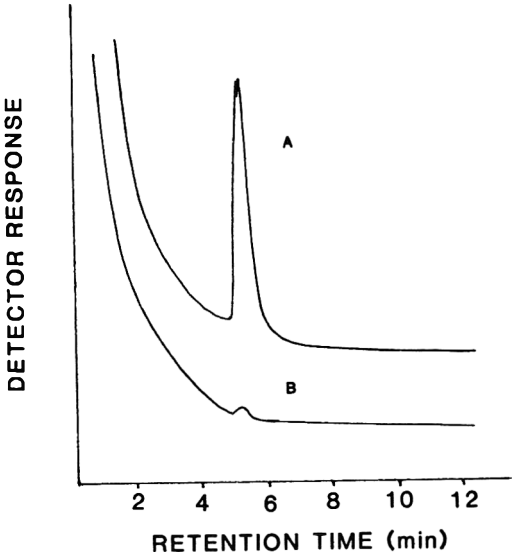

Figure 1. Chromatogram of (A) swine liver fortified with 0.1 ppm sulfamethazine, and (B) control swine liver.

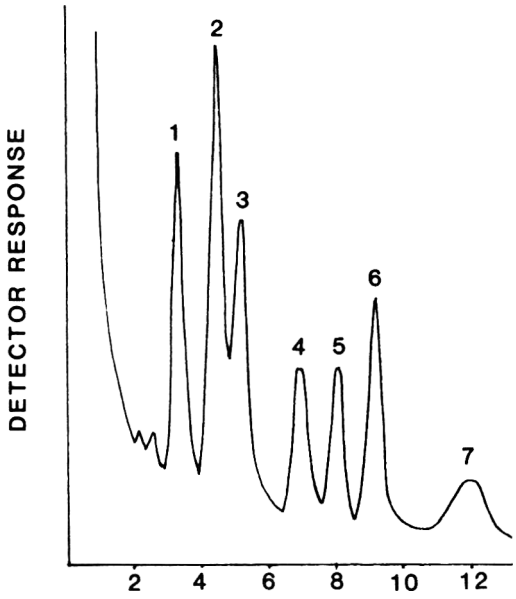

Figure 2. Chromatogram showing resolution of swine liver fortified with 0.1 ppm of: (1) sulfathiazole, (2) sulfachloropyrazine, (3) sulfamethazine, (4) sulfaethoxypyridazine, (5) sulfadimethoxine, (6) sulfabromomethazine, and (7) sulfaquinoxaline.

(fortification range in liver of 0.1–1.0 ppm) were performed with 6 other sulfonamides, using the acid hydrolysis procedure. The average values and the ranges were as follows: sulfathiazole, 67.8% (53.5–85.0%); sulfachloropyrazine, 103% (91.2–117%); sulfaethoxypyridazine, 78.3% (59.5–100%); sulfadimethoxine, 81.9% (61.0–106%); sulfabromomethazine, 89.2% (73.5–106%); and sulfaquinoxaline, 73.4% (55.5–94.0%). The reason for the somewhat low and variable recoveries obtained for some of these sulfonamides is not known.

Table 3. Determination of sulfamethazine residues (ppm) in swine liver and kidney by GLC and colorimetric procedures

Days after withdrawal	Liver		Kidney	
	GLC	Color	GLC	Color
0	4.22	2.88	3.72	2.21
0	4.88	2.52	3.47	1.59
0	4.32	1.95	4.11	1.87
0	4.59	3.43	4.30	2.49
0	5.21	3.33	4.50	1.87
7	0.10	0.11	<0.10	<0.10
7	0.12	0.12	<0.10	<0.10
7	<0.10	0.15	<0.10	<0.10
7	<0.10	<0.10	<0.10	<0.10
7	0.21	0.20	0.12	0.13
10	<0.10	<0.10	<0.10	<0.10
10	<0.10	<0.10	<0.10	<0.10
10	<0.10	<0.10	<0.10	<0.10
10	<0.10	<0.10	<0.10	<0.10
10	<0.10	<0.10	<0.10	<0.10
14	<0.10	<0.10	<0.10	<0.10
14	<0.10	<0.10	<0.10	<0.10
14	<0.10	<0.10	<0.10	<0.10
14	<0.10	<0.10	<0.10	<0.10
14	<0.10	<0.10	<0.10	<0.10

Continuing work to replace diazomethane as the methylating reagent has indicated that MethElute™ methylating reagent (Pierce Cat. No. 49301) may serve this purpose. This material is an on-column methylating agent and is used as received from the manufacturer by adding 5 μL/mL reagent to the sample extract before injection onto the GC column. Additional work is in progress to determine whether this material is completely satisfactory.

Acknowledgments

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COSMETICS

High Pressure Liquid Chromatographic-Thermal Energy Determination of *N*-Nitrosodiethanolamine in Cosmetics

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Methods for the determination and confirmation of *N*-nitrosodiethanolamine (NDELA) in cosmetic products were developed. The NDELA fraction was isolated from a cosmetic product by a series of solvent extractions which were designed to concentrate the NDELA and remove ingredients deleterious to the analytical system. The isolated fraction was then analyzed for NDELA using a high pressure liquid chromatograph (HPLC) interfaced with a thermal energy analyzer (TEA). The compound was measured by comparison of detector response with those of known standards. NDELA was verified by gas chromatography-mass spectrometry of the silyl derivative after preliminary cleanup of the sample by gradient elution HPLC on a Partisil 10 PAC column. The limit of detection of NDELA by TEA is 2-3 ng, which corresponds to 20-30 ppb in the cosmetic product. Analysis of an emulsion cream and a hair grooming gel spiked at 3 and 4 ng concentration levels, respectively, yielded recoveries ranging from 71 to 103% (average 88%).

The widespread occurrence of *N*-nitrosamines in the environment is now well recognized (1-3). Nitrosamines have been found in soil (4), water (5), herbicides (6), and air (7). Crosby et al. (8) reported the presence of *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine in some foods preserved with nitrite, most notably cooked bacon. In 1977, Fan et al. (9) reported that *N*-nitrosodiethanolamine (NDELA) was present as a contaminant in industrial cutting fluids that had been formulated with sodium nitrite and triethanolamine. NDELA was also found in unburned processed tobacco that had been treated with the herbicide maleic hydrazide formulated as the diethanolamine salt (10). Lijinsky et al. (11) demonstrated that NDELA could be formed easily by nitrosation of either di- or triethanolamine. The carcinogenic activity of NDELA in rats was reported by Druckrey et al. (1).

Fan et al. (12) reported that NDELA was present in some cosmetics such as lotions and shampoos. The NDELA precursors, di- and triethanolamine, are widely used in cosmetics. For example, diethanolamine is a component of some commercial grades of triethanolamine and a large number of diethanolamine salts and amides of fatty acids which are used as foam boosters, stabilizers, and conditioners in shampoos and related products. Also, when alkanolamines are combined with fatty acids, they form a soap that may serve as an emulsifier for many types of cosmetic creams and lotions. The levels of NDELA reported by Fan et al. were as high as 45 000 ppb, although a majority of the samples contained a much lower level of this contaminant. Fan et al. reported that recoveries of NDELA added to a variety of cosmetic products ranged from 7 to 103%.

In our laboratory (J. A. Wenninger, unpublished results, 1980), 335 cosmetic products have been analyzed for NDELA contamination, using the methodology reported by Fan et al. (9) for some products and the method described in this paper for others. The results indicated that 7% of the products were contaminated with NDELA at levels greater than 2000 ppb, 28% at levels between 30 and 2000 ppb, and 7% at trace levels (10-30 ppb). No NDELA was detected in 58% of the products analyzed. The NDELA was confirmed by gas chromatography-mass spectrometry (GC-MS) in cosmetic products that were contaminated at levels greater than 1000 ppb. The highest levels of NDELA contamination were associated with cosmetic products that contained the preservative 2-bromo-2-nitro-1,3-propanediol (BNPD) and either diethanolamine, triethanolamine, or a derivative of these ingredients. Schmeltz and Wenger (13) reported on the indirect nitrosation of diethanolamine by BNPD.

Results of the analyses of cosmetic ingredients for NDELA and trace level "nitrite" contamination have been reported (J. H. Merritt, Cosmetic, Toiletry and Fragrance Association, Washington, DC 20005, unpublished results, 1980). Cosmetic raw materials in general contributed little NDELA to cosmetic formulations and ethanola-

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mines were relatively free of NDELA contamination. However, condensation products of ethanolamines with fatty acids appeared to be a potential source of low levels of NDELA in cosmetic products. Results of the analyses of cosmetic ingredients for trace levels of "nitrite," with the possible exception of sodium lauryl sulfate, indicated that contaminants of cosmetic ingredients are not a significant source of nitrosating agents.

Using the procedure described by Fan et al. (12), we sometimes were unable to obtain reproducible results on duplicate analyses and experienced variations in results when known amounts of NDELA were added to samples. Low recoveries were usually associated with samples that contained relatively high proportions of water. The presence of water apparently caused the initial extraction with ethyl acetate to be incomplete and the subsequent chromatographic separation to be erratic. Although the ethyl acetate extract was filtered through anhydrous sodium sulfate before chromatographic separation, contact time with this drying agent was insufficient to effectively remove water. After modification of the Fan et al. procedure, principally removing water from the sample before extraction and minimizing the adsorption of NDELA on glassware surfaces, improved recoveries were obtained.

The procedure described in this paper involves a series of solvent extractions designed to isolate NDELA along with other ingredients of similar solubility and polarity. The extract is then analyzed by the high pressure liquid chromatographic-thermal energy analyzer (HPLC-TEA) system. The method was verified by conducting recovery studies using cosmetic products containing known levels of NDELA.

METHOD

Apparatus

(a) *Nitrosamine detector.*—TEA Model 502 (Thermo Electron Corp., Waltham, MA 02154).

(b) *HPLC pump.*—Varian Model 8500 (Varian Associates, Palo Alto, CA 94303), or equivalent.

(c) *Injection valve.*—Equipped with 20 μL sample loop and capable of operation at 6000 psi (Model 7120, Rheodyne, Inc., Berkeley, CA 94710), or equivalent.

(d) *HPLC guard column.*—75 × 4.6 mm id stainless steel with 10 μm frits packed with Corasil Type II, 37–50 mm (Waters Associates, Milford, MA 01757).

(e) *HPLC analytical column.*—250 × 4.6 mm id stainless steel packed with 10 μm silica gel (Partisil® 10 PAC, Whatman Inc., Clifton, NJ 07014), or equivalent.

(f) *Strip chart recorder.*—Model 7127 (Hewlett-Packard, Palo Alto, CA 94304), or equivalent.

Connect, in series, the HPLC pump, injector, in-line filter, guard column, HPLC column, and TEA using appropriate fittings and $^1/_{16}$ in. od stainless steel tubing. If recorder does not have 10 V input, use appropriate voltage divider between TEA and recorder (voltage divider can be installed by Thermo Electron Corp.).

Operating conditions: HPLC eluant hexane–acetone (1 + 1) at 120 mL/h; TEA carrier gas argon, adjust flow rate to obtain pressure of 1 torr; oxygen flow rate, adjust to obtain increase of pressure from 1 to 1.5 torr; TEA catalytic furnace temperature 55°C; TEA cold trap, fill 12 cm id Dewar flask with slush of powdered dry ice–ethanol.

(g) *Freeze dryer.*—Centrifugal Bio-Dryer (VirTis Co., Inc., Gardiner, NY 12525), or equivalent.

(h) *Chromatographic tubes.*—60 × 2.2 cm id, with stopcock.

(i) *High pressure liquid chromatograph.*—Equipped with ultraviolet (UV) detector and gradient elution capability. Use HPLC column (e), or equivalent. Waters Model 244 with 2 Model 6000 A solvent pumps, U6K universal injector, 440 UV detector equipped with 254 nm filter and 660 solvent programmer (Waters Associates), or equivalent. Operating conditions: solvent program, linear, 15 min, from 80 to 20% solvent A; solvent flow rate 2 mL/min.

(j) *Strip chart recorder.*—10 in., equipped with 10 mV input (Model A-25, Varian Associates), or equivalent.

(k) *Gas chromatograph–mass spectrometer.*—Model 5992A (Hewlett-Packard), or equivalent. Gas chromatograph is equipped with 150 cm × 2 mm id glass column packed with 3% SP-2100 on 100–120 mesh Chromosorb® W (HP).

Operating conditions: peakfinder program; temperatures (°C): column programmed from 114 to 280 at 4°/min, injection port 200; carrier gas (helium) flow rate 20 mL/min; MS peak detection threshold 1000; samples/0.1 amu 4; electron multiplier 1200; GC peak detection threshold 10; single ion peak trigger 116 amu.

(l) *Test tubes.*—150 × 20 mm Pyrex®. Treat with silanizing solution 5 min. Rinse with toluene; then methanol.

(m) *UV lamp.*—Mineralight Model UVSL.25

(Ultra-Violet Products, Inc., San Gabriel, CA 91778), or equivalent.

Reagents

(a) *Solvents.*—Ethyl acetate, hexane, acetone, methylene chloride, methanol, and 2,2,4-trimethylpentane (Distilled-in-Glass, Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442), or equivalent.

(b) *NDELA.*—Supplied by Battelle Columbus Laboratories, Columbus, OH. Purity and identity of NDELA standard were verified by HPLC and GC-MS. *Stock solution 1.*—Accurately weigh 25 mg NDELA and transfer, with aid of acetone, to 100 mL volumetric flask. Dilute to volume with acetone and mix. *Stock solution 2.*—Pipet 5.0 mL stock solution 1 into 100 mL volumetric flask, dilute to volume with acetone, and mix. *Working solution 1.*—Pipet 3.0 mL stock solution 2 into 25 mL volumetric flask, dilute to volume with acetone, and mix. *Working solution 2.*—Pipet 1.0 mL stock solution 2 into 25 mL volumetric flask, dilute to volume with acetone, and mix.

Cover containers of stock and working solutions with aluminum foil to protect contents from light. Store solutions in refrigerator. Prepare working solutions fresh weekly.

(c) *N,O-bis-(Trimethylsilyl)-trifluoracetamide (BSTFA).*—No. 38828, Pierce Chemical Co., Rockford, IL 61105.

(d) *Trichloromethylsilane.*—No. 9646, Eastman Organic Chemicals, Rochester, NY 14650.

(e) *Dichlorodimethylsilane.*—No. 9650, Eastman Organic Chemicals.

(f) *HPLC solvent for NDELA determination.*—Hexane–acetone (1 + 1). Degas and filter.

(g) *HPLC solvents for NDELA confirmation.*—*Solvent A.*—2,2,4-Trimethylpentane–acetic acid (500 + 2). *Solvent B.*—Methylene chloride–methanol–acetic acid (450 + 50 + 2). Degas and filter.

(h) *Filter aid.*—Acid-washed Celite 545 (Johns-Manville, Denver, CO 80217). To ca 700 g Celite in 4 L beaker add 3 L 20% HCl and stir. Heat on steam bath 3–4 h, stirring occasionally. Filter through Buchner funnel with suction. Wash with water until washings are iron- and chloride-free. Suction filter cake dry, transfer to beaker, and dry in 135°C oven.

(i) *Silanizing solution.*—Toluene–trichloromethylsilane–dichlorodimethylsilane (69 + 2 + 2).

Preparation of Sample

Accurately weigh ca 5 g sample into tared 150 mL beaker. Add 250 mg ammonium sulfamate, 20 g Na_2SO_4, and ca 10 g Celite, and stir until mixture is uniform. Let stand ≥4 h to let Na_2SO_4 remove water. Place small plug of glass wool in bottom of chromatographic tube and add sample mixture. Pack lightly with tamper. Add 80 mL hexane to column. Discard collected eluate. Add 125 mL ethyl acetate to column, collecting eluate in 250 mL beaker. Add 2 drops of propylene glycol to ethyl acetate extract and concentrate to 3–4 mL under slow stream of nitrogen. Transfer, with aid of several small portions of ethyl acetate, to tapered 50 mL centrifuge tube. Add 1 mL water, shake well, and, after phases have separated, remove aqueous layer with small pipet or syringe. Repeat extraction twice more. If emulsion forms because of presence of surfactants, evaporate ethyl acetate to dryness in 50 mL beaker. Extract residue with 3–4 small portions (ca 1 mL) of water. Place combined aqueous extracts in silanized test tube and freeze-dry. Extract residue with 2–3 small portions (ca 1 mL) of acetone, transferring extracts to tared screw-cap vial. Evaporate to dryness under nitrogen, and weigh. Cap tightly and refrigerate residue. Before HPLC-TEA analysis, prepare analytical sample by adding 1.0 mL acetone to dissolve residue.

Determination

Place HPLC-TEA into operation, using manufacturer's instructions and conditions outlined under *Apparatus.* When baseline has stabilized, inject 20 μL Working Solution 1 at ×8 or ×16 attenuation to check system function. Slightly alter carrier gas flow and again inject standard, noting detector response. Repeat until response has been maximized. Using same procedure, adjust oxygen flow rate to obtain maximum response.

After detector response has been maximized and baseline is stable, inject 20 μL sample at ×8 attenuation. If response is obtained at same retention as NDELA standard, determine which NDELA standard will give a response most nearly the same as the sample. After correct standard has been selected, inject standard and then sample, followed by another standard. Purge cold trap and, after baseline has stabilized, repeat analyses to obtain second set of analytical data.

Calculation

For each set of data, measure peak heights of standards and calculate average. Measure sample peak height and calculate NDELA content as follows:

$$\text{NDELA (ppb)} = (V_u \times C_s \times V_{is} \times PH_u \times A_u)/(PH_s \times A_s \times V_{iu} \times W_u)$$

where V_u = volume of analytical sample, μL, i.e., equals volume of acetone added, μL, plus wt of freeze-dried residue, mg (assume sample density to be 1.0 g/mL); C_s = concentration of NDELA standard, ng/μL; V_{is} = volume of standard injected, μL; PH_u = peak height of NDELA in sample, arbitrary units; A_u = attenuation of NDELA sample peak; PH_s = peak height of NDELA in standard, arbitrary units; A_s = attenuation of NDELA standard peak; V_{iu} = volume of sample injected, μL; W_u = weight of gross sample, g.

If Rheodyne injection valve with 20 μL sample loop is used, eliminate V_{is} and V_{iu} from above equation.

Ultraviolet Confirmatory Test

Transfer portion of acetone solution of sample (ca 100 μL) to small glass vial, cap, and place within several inches of shortwave UV source. After exposure overnight, re-analyze by HPLC-TEA. Disappearance of peak corresponding to NDELA is good evidence of its presence.

Gas Chromatographic-Mass Spectrometric Confirmation

Further confirmation of NDELA may be obtained as follows: Using conditions given under *Apparatus* (**i**), inject 20–30 ng NDELA standard with UV detector at 0.20 absorbance unit full scale. Determine retention time of standard NDELA. Next, inject 50–75 μL analytical sample, using absorbance setting appropriate for amount of NDELA previously determined to be present. Collect eluting NDELA peak in 50 mL silanized tapered centrifuge tube. Repeat until 0.5–1 μg NDELA has been collected. Evaporate solvent in 40°C oil bath under slow stream of dry nitrogen. When volume is ca 400 μL, transfer to small vial and continue evaporation to dryness at room temperature. Add 250 μL BSTFA, tightly cap, and warm 10 min at 75°C. To prepare standard, add 4–5 μL Stock Solution 1 to small vial, evaporate to dryness, and prepare silyl derivatives as above. Store solutions in desiccator protected from light. Analyze solutions by GC-MS within 24 h.

Using conditions outlined under *Apparatus* (**k**), inject 1–2 μL standard solution and obtain mass spectrum of NDELA silyl derivative. Repeat with sample solution, and compare mass spectrum with that of standard for confirmation.

Results and Discussion

NDELA can be detected and determined by using HPLC or GC in conjunction with a wide variety of detection systems. In our laboratory the HPLC-TEA combination, although it has some disadvantages, is used because of the high specificity and sensitivity of the detector system for nitrosamines and because the technique does not require derivatization of NDELA. This is especially important for cosmetics because of the large number of potential interfering substances (e.g., fragrance ingredients, emulsifiers, preservatives, etc.) which vary widely from one product to another.

Before the development of the proposed sample preparation technique, it was decided to eliminate the use of silica gel column chromatography because of losses experienced in previous recovery studies. Because of the inherent specificity of the HPLC-TEA combination, direct interferences in the determination of NDELA were not expected. Any potential interfering substances, if present, would not be removed by employing an adsorption column inherently less efficient than the analytical column used for HPLC-TEA analysis. Therefore, our aims in the development of the sample preparation procedure were only to concentrate the NDELA fraction and to remove ingredients, such as pigments and ionic compounds, that would be deleterious to the analytical HPLC column.

In the initial sample preparation step, anhydrous sodium sulfate was mixed with the sample to remove water. Celite was then mixed with the sample to hold any pigments and to aid the flow of extraction solvents. Frequently, however, a separation into 2 phases occurred when the ethyl acetate eluate was concentrated. For this reason, it was necessary to add the water and acetone extraction steps. The adsorption of NDELA on glassware surfaces was minimized by silanization of glassware and addition of propylene glycol to compete for adsorption sites. Studies to evaluate the effect that these procedures had on recoveries were carried out. Silanization of the test tubes increased recoveries from an average of 31 to 84%. Propylene glycol improved recoveries by approximately 10%. Drying agents more efficient than anhydrous sodium sulfate should not be used in any part of the procedure. When very efficient drying agents, such as calcium sulfate, were used to remove traces of water, losses of added NDELA by adsorption on glass surfaces were severe or total.

During the analysis of cosmetic products, false

Table 1. Recovery of NDELA from an emulsion cream and a hair grooming gel

Sample	Added, ng/g (ppb)	Found, ng/g (ppb)	Rec., %
Emulsion cream	362	290	80
	362	338	93
	362	331	91
	290	261	90
	290	258	89
	290	206	71
	120	115	96
	120	94	78
	120	101	84
Av. rec. (N = 9)			86
SD (σ)			±6.4
Hair grooming gel	362	278	77
	362	311	86
	362	284	78
	290	267	92
	290	299	103
	290	249	86
	120	96	80
	120	104	87
	120	115	96
	97	86	89
	97	90	93
	97	96	99
Av. rec. (N = 12)			89
SD (σ)			±8.2

positives were occasionally encountered. The false positives were not interfering compounds, but resulted from the desorption of previously adsorbed NDELA from the HPLC column by water contained in the sample. If a sample contains relatively large amounts of polar compounds, such as triethanolamine or glycerol, removal of the last traces of water by freeze-drying is difficult. In cases where false positives are suspected they are easily recognized because they fail the UV confirmatory test. When a false positive has been identified, the sample solution should be dried with sodium sulfate and reanalyzed. An alternative viable approach to the problem of false positives is the substitution of normal phase partition HPLC columns in place of the silica gel column. The bonded cyano or amino columns are applicable to this procedure and do not adsorb detectable amounts of NDELA. Our experience using these columns, however, is that more frequent cleaning is required. Reverse phase HPLC columns should not be used in conjunction with the TEA because aqueous eluants cause the instrument's response to nitroso compounds to be low and erratic.

Recovery studies were conducted by the method described using 2 commercial cosmetic formulations: an emulsion cream and a protein hair grooming gel. Neither product contained any NDELA precursors and analysis of each showed the absence of NDELA at the 20–30 ppb limit of detection. Known amounts of NDELA standard were added to 5 g samples of the cosmetics, mixed, and then analyzed by the proposed procedure. Recovery studies were conducted at levels of 120, 290, and 362 ppb for each product. Studies at 97 ppb were performed on the hair grooming gel. Results are shown in Table 1.

In conclusion, the described method for the HPLC-TEA determination of NDELA has been demonstrated to give reliable and reproducible results for a large number of cosmetic products having widely different compositions.

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DAIRY PRODUCTS

High Performance Liquid Chromatographic Determination of Lactose in Milk

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A rapid and simple high performance liquid chromatographic method for the determination of lactose in milk was developed. Samples were diluted with 0.5% perchloric acid and centrifuged, and an aliquot of the supernate was mixed with acetonitrile. Lactose was separated on a 10 μm particle-size silica column with aqueous acetonitrile as the mobile phase. The recovery of lactose from whole, skim, and chocolate milk averaged 99.2, 101.1, and 100.4%, respectively. Coefficients of variation for routinely performed duplicate determinations are between 1.0 and 1.5%.

High performance liquid chromatography (HPLC) offers many advantages to the analyst for the determination of lactose in milk. The most obvious include speed, reliability, and simplicity of sample preparation. The flexibility of the instrumentation to perform other analyses is also an advantage for laboratories whose sample load does not justify an instrument dedicated to lactose analysis.

In spite of these benefits, only a few studies have been reported using HPLC for determining lactose in milk. Hobbs and Lawrence (1) selected a stationary phase of strongly acidic cation exchange resin and aqueous ethanol as their mobile phase to analyze previously defatted milk samples. Sample preparation was minimal, and total analysis time for replicates was 1 h with the bulk of this time taken up by lengthy column retention of lactose. Unless several chromatographic instruments were used, a backlog of unanalyzed samples could rapidly result. The authors speculated on a quicker chromatographic system, but no experimentation was presented. Conrad and Palmer (2) suggested mixing milk with water followed by dilution with acetonitrile before chromatography on a μBondapak/carbohydrate® column (Waters Associates, Inc., Milford, MA 01757) using aqueous acetonitrile as the mobile phase. Although the chromatography was rapid (less than 3 min), samples had to stand several hours to allow precipitation of fat and protein before analysis. Difficulty in using this system for quantitation of lactose in milk was reported by Warthesen and Kramer (3), yet quantitation could be achieved if absolute ethanol was substituted for acetonitrile as the diluant. DeVries et al. (4) included milk samples in their evaluation of HPLC methodology as an alternative to official procedures for carbohydrate analysis in foods. Bonded phase columns, such as μBondapak/carbohydrate, were again selected for the chromatography, and the extraction procedure consisted of mixing the food sample with water followed by dilution with acetonitrile. Unfortunately, little detail was given for milk analysis and a limited number of samples appear to have been analyzed. Euber and Brunner (5) also used the μBondapak/carbohydrate column, aqueous acetonitrile mobile phase, and initial dilution of milk with water, but samples were deproteinized by treatment with trichloroacetic acid before chromatography. An analysis could be completed in 45 min.

The method developed in our laboratory involves extraction of lactose from milk and removal of fat and protein with dilute perchloric acid followed by centrifugation. An aliquot of the supernate is mixed with acetonitrile and chromatographed on unmodified 10 μm particle-size silica, using aqueous acetonitrile as the mobile phase. The lactose concentration is then calculated by comparison with external standards. Total analysis time is about 30 min.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Model 5010, differential refractometer, Model 220L chromatography computer (Varian Instrument Div., Palo Alto, CA 94303) and a loop valve injector (Valco Instrument Co., Houston, TX 77024) with 100 μL loop. A Haake Model FE water bath (Haake Inc., Saddle Brook, NJ 07662) maintained the detector at 30 ± 0.01°C. Recorder Model 555 (Linear Instruments Corp., Irvine, CA 92714).

(b) *Chromatographic column.*—30 cm × 4 mm Micropak® Si-10 (Varian) or equivalent.

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(c) *Guard column.*—4 cm × 4.6 mm (Upchurch Scientific, Sunnyvale, CA 94086) or equivalent packed with HC Pellosil® (Whatman Inc., Clifton, NJ 07014) or equivalent.

Reagents

(a) *Solvents.*—HPLC grade acetonitrile and water (J. T. Baker Co., Phillipsburg, NJ 08865) or equivalent.

(b) *Mobile phase.*—Acetonitrile + water (80+20). Pass through 47 mm 0.45 μm filter (Millipore Corp., Bedford, MA 01730).

(c) *Perchloric acid.*—0.5%. For each 1 L, dilute 7.2 g AR grade 70% perchloric acid (Mallinckrodt, Inc., St. Louis, MO 63147) with water.

(d) *Lactose standards.*—Prepare 60 mg/mL standard by dissolving 3.00 g AR grade lactose·H_2O (Mallinckrodt, Inc.) in 50 mL water. Transfer 8, 6, and 4 mL aliquots to 10 mL volumetric flasks and dilute to volume to obtain 48, 36, and 24 mg/mL standards, respectively. Use 1 mL of each standard in place of milk sample as next described.

Sample Preparation

Transfer 1 mL milk sample into previously tared centrifuge tube and record weight of milk. Add 20 mL 0.5% perchloric acid and vigorously shake or vortex tube. Centrifuge 20 min at ca 3000× g. Remove 1 mL supernate and place in glass tube. Add 4 mL acetonitrile, and vortex. Pass through 13 mm 0.45 μm filter (Millipore Corp.) or Acrodisc-CR® (Gelman, Ann Arbor, MI 48106).

Liquid Chromatography

Equilibrate system with mobile phase ca 1 h. Flow rate for equilibration and analysis is 2.0 mL/min. Inject 100 μL of each standard followed by samples.

Calculations

Perform linear regression analysis of standard peak areas and concentrations (mg standard lactose·H_2O added to each centrifuge tube, i.e., 60, 48, 36, and 24 mg). From regression line, calculate mg lactose·H_2O in each 1 mL milk sample. Divide by sample weight in mg and multiply by 100 to obtain % lactose·H_2O by weight.

Results and Discussion

Chromatograms of several milk extracts are presented in Figure 1. Lactose was eluted in about 5 min and its resolution from sucrose present in chocolate milk, although not quite at baseline, was more than adequate for reliable quantitation. We used a 30 cm × 4 mm column containing 10 μm irregular particle-size silica for our work, but equally good results can be obtained with a similar packing in the more common 25 cm × 4.6 mm column. Although smaller bore columns (e.g., 25 cm × 2.6 mm) yielded acceptable results with reduced flow rates, the larger bore columns were preferred for maximum efficiency and speed with this method.

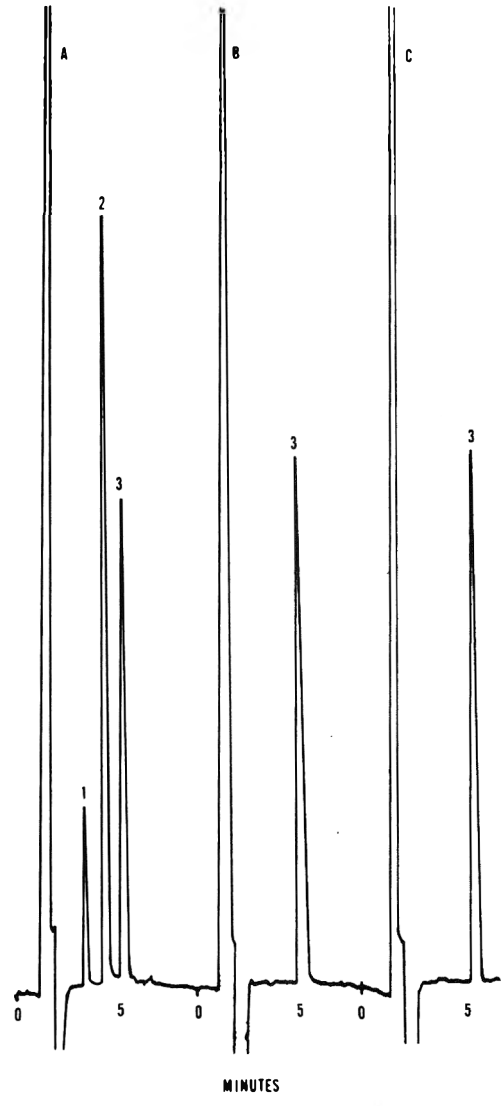

Figure 1. Liquid chromatograms of milk extracts: A, chocolate; B, whole; C, skim; 1, fructose + glucose; 2, sucrose; 3, lactose.

Although the supernate does not have to be clear after centrifugation, this initial removal of

part of the protein with 0.5% perchloric acid treatment was necessary for easy filtration of the final solution. Lactose, by itself or in milk, was stable even if allowed to stand more than 4 h in 0.5% perchloric acid or acetonitrile–0.5% perchloric acid (4+1). Because sucrose undergoes some hydrolysis, this method should not be used for its quantitation. Concerns over the safety of acidified acetonitrile (6) were unfounded. Mixtures of acetonitrile–0.5% perchloric acid (4+1) were tested for the evolution of hydrogen cyanide by using sodium picrate papers (7). No color change was observed on the test strips held in sealed tubes at room temperature over 5 h.

Samples and standards contained lactose at levels which did not precipitate when diluted with acetonitrile in the final step. To determine lactose solubility, known amounts of this sugar were dissolved in water and then diluted with sufficient acetonitrile to obtain the 4+1 acetonitrile–water mixture. Lactose at concentrations greater than 10 mg/mL in water precipitated almost immediately when acetonitrile was added. Slow crystallization of lactose occurred at 10 mg/mL, but at 5 mg/mL and less remained clear, even with overnight standing. These findings may explain the poor recoveries previously mentioned for the method of Conrad and Palmer (2). Their extracts contained almost 25 mg/mL lactose before dilution with acetonitrile, a concentration which could precipitate immediately.

We do not recommend injecting aqueous solutions of lactose directly into the liquid chromatograph. Unless sufficiently dilute, lactose could precipitate when rapidly mixed with the mobile phase. As discussed by DeVries et al. (4), injection of carbohydrates dissolved in a solvent stronger than the mobile phase can also adversely influence the quality of chromatography. Severe band broadening and lessened detector response were observed. Euber and Brunner (5) likewise reported a loss of resolution and peak broadening with aqueous lactose injections exceeding 30 μL. Prior dissolution of samples in the mobile phase (or as close to it as possible) essentially eliminates these problems while not adding significantly to preparation time.

Recovery studies were performed as given in Warthesen and Kramer (3), except that the sample size was decreased by one-third rather than one-half. Recovery of lactose dissolved in duplicate whole, skim, and chocolate milk samples averaged 99.2, 101.1, and 100.4%, respectively. As indicated by these data, the added sucrose present in chocolate milk did not result in precipitation of lactose when analyzed by this method. The homogeneity of the lactose peak was determined with extracts prepared from lactase (Sigma Chemical Co., St. Louis, MO 63178)-treated milk. No interfering peaks were detected at the elution volume for lactose. In our laboratory, we routinely perform duplicate determinations of milk samples, obtaining coefficients of variation between 1.0 and 1.5%. The coefficient of linear regression for the standards was consistently 0.99. By using automatic dispensing devices wherever possible, the total analysis time was only 30 min.

We believe our method offers several advantages over established procedures for the determination of lactose in milk and is worthy of collaborative study.

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COLOR ADDITIVES

Spectrophotometric Determination of Phosphorus in Certifiable Straight Color Additives: Collaborative Study

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A simple and rapid spectrophotometric method was developed for determining the total phosphorus content of certifiable straight color additives. The dye sample is mixed with a cellulose powder and MgO mixture, and ashed at 500°C in a small Pyrex beaker in a muffle furnace. The ash is dissolved in vanadomolybdic acid reagent and filtered through glass wool, and the absorbance of the resulting yellow molybdovanadophosphoric acid solution is measured at 400 nm. The total phosphorus content of the sample, expressed as percent Na_3PO_4, is determined from a standard curve. Recovery of phosphorus added as KH_2PO_4 to 39 different dyes in amounts equivalent to 0.300% Na_3PO_4 ranged from 95.3 to 106.8%, averaging 100.6%. In the collaborative study, 7 laboratories successfully performed duplicate analyses of 6 different dyes (D&C Orange No. 5, D&C Yellow No. 8, FD&C Blue No. 2, FD&C Red No. 3, FD&C Red No. 40, and FD&C Green No. 3). The mean values found ranged from 0.325 to 6.86% Na_3PO_4. In general, the accuracy and reproducibility of the method were satisfactory, with single determination coefficients of variation ranging from 3.76 to 9.60%. The method was adopted official first action.

In the manufacture of color additives, Na_3PO_4 is often added to precipitate any Pb that may be present. Occasionally dyes are found that contain phosphate, probably derived primarily from this source. Quantitative data on the amount of phosphate present is desirable for use in the FDA certification program so that it may be combined with various other determinations such as percent pure dye, volatile matter, NaCl, Na_2SO_4, etc., to account for the entire sample. The phosphate content of the water-soluble dyes is also of interest because it interferes with the determination of Na_2SO_4 by potentiometric titration (1).

In the determination of phosphorus by spectrophotometric methods, it is first necessary to remove organic matter that would interfere with absorbance readings. This may be done by wet-ashing with acids such as HNO_3, H_2SO_4, or $HClO_4$, or by dry-ashing with a gas burner or in a muffle furnace. In the method reported here, dye samples were ashed in a muffle furnace.

A number of spectrophotometric methods are available, but the one selected is preferable to some used widely in the past, such as the molybdenum blue method (2) because of the greater stability of the color complex, simplicity, and relative freedom from interferences. The molybdovanadophosphate method used was proposed by Misson (3) in 1908. It was first employed in an official AOAC method in 1957 for determining total phosphorus in fertilizers (4), and has since been used in several other official methods. The author, using dry-ashing to prepare the samples, modified Misson's method for use in analyzing fertilizers (5), fruits and fruit products (6), dairy products (unpublished report), and eggs and egg products (unpublished report). The method for fruits and fruit products was adopted as an official AOAC method (7).

Development of Method

Because no method was available for the determination of phosphorus in dyes, a project was instituted to develop one. The main problem in applying the molybdovanadophosphate method to the analysis of certifiable straight color additives was developing a satisfactory dry-ashing procedure. The ashing technique finally developed is very simple, and works well with all straight dyes, whereas earlier versions did not. The method was also applied to lakes, but the results were unsatisfactory.

Initially, platinum dishes were tried but they became stained, and may have been attacked chemically, by some of the dyes. The stain was

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The recommendations of the Associate Referee were approved by the General Referee and Committee G and were adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1981) 64, 443

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very difficult to remove. Next, Erlenmeyer flasks were tried (first the 25 mL size, then the 50 mL and 125 mL sizes) in an attempt to improve ashing, but they were unsatisfactory because of the excessive amount of unashed material remaining even after several hours at 600°C. Next, 250 mL Pyrex beakers were tried. These gave much better ashing than Erlenmeyer flasks, but they were soon replaced by 100 mL beakers, which were equally satisfactory and allowed more samples to be ashed at the same time, because less space was required.

In the early work on this method, the dye samples were dissolved in an aqueous solution of $Mg(NO_3)_2$ or MgO and HNO_3, and evaporated to dryness on a hot plate before ashing. This was done to hydrolyze any nonorthophosphates present to orthophosphates (the only form the method will measure) and convert them, and any monobasic or dibasic phosphates present, to heat-stable $Mg_3(PO_4)_2$. Since some samples were difficult to ash, sucrose was incorporated in the ashing solution; ashing was improved, but still insufficient. Initially, samples were ashed in the muffle at 500°C; later 550°C and 600°C were used to improve ashing until it was observed that the ash of some types of samples etched the bottom of the beaker or left a slight deposit on it. This was not considered serious until it was discovered that these beakers sometimes produced erroneously high results when re-used.

A satisfactory procedure resulted from the use of a mixture of cellulose powder and MgO. Etching was prevented, and the same beakers were used repeatedly without evidence of cross-contamination. With most types of samples, very little carbon remained after ashing 3 h at 500°C, and this caused no significant error. However, several types of samples could not be ashed satisfactorily, even at higher temperatures and for much longer times. Low recoveries were obtained on samples with carbon remaining in the sample ash. A reduction in the residual carbon and good recoveries were obtained by ashing a second time after mixing cellulose powder with the ash.

In the planning of this work, an acid hydrolysis step was considered necessary to convert nonorthophosphates that might be present to orthophosphates. Recovery experiments using sodium metaphosphate and sodium pyrophosphate indicated that a conversion step was unnecessary in the proposed method, probably because water and acids produced during the ashing with cellulose and MgO effected the necessary conversions. Therefore, the method measures the *total* phosphorus content of the sample.

The cellulose powder appears to perform the following functions: (*1*) facilitates uniform mixing of the small amount of MgO with the dye, (*2*) promotes hydrolysis of nonorthophosphates, (*3*) facilitates reaction of highly insoluble MgO with phosphates, (*4*) greatly increases the rate and completeness of ashing, and (*5*) prevents etching of the beaker.

Experimental

Recovery studies were performed on 39 different straight color additives submitted for certification. They were analyzed by the proposed method and contained 0.000–0.899% phosphorus expressed as Na_3PO_4. Then they were spiked with a solution of KH_2PO_4 to obtain the equivalent of 0.300% added Na_3PO_4 and analyzed by the proposed method, and the percent recovery was calculated. Recoveries ranged from 95.3 to 106.8%, averaging 100.6% as shown in Table 1.

To measure the precision of the method, 6 dyes, selected to represent a range of phosphorus content, were analyzed in duplicate on 5 different days. The results given in Table 2 show very good agreement between duplicate determinations and good agreement among the values obtained on different days. The ranges covered by the 10 values for each dye were as follows: FD&C Green No. 3, 0.895–0.935; D&C Yellow No. 8, 0.772–0.811; D&C Red No. 21, 0.474–0.518; FD&C Blue No. 2, 0.299–0.314; D&C Red No. 33, 0.228–0.239; D&C Green No. 5, 0.140–0.156 (values expressed as percent Na_3PO_4).

Table 3 presents recovery data obtained with dyes found difficult to ash. The first column contains the recovery results obtained when carbon remained after ashing, and the second column contains recovery results obtained when this carbon was removed by re-ashing after mixing the ash with cellulose powder. Recoveries obtained after the usual ashing were very low, ranging from 12.9 to 94.1%; recoveries obtained after re-ashing were good, ranging from 97.1 to 104.0%.

Collaborative Study

Three practice samples with the phosphate content stated and 12 blind duplicate samples of unspecified phosphate content representing 6 different certifiable straight color additives were sent to 7 collaborating laboratories. Collaborators were also sent an instruction sheet, a copy of the method, and 5 sheets for reporting results.

Table 1. Recovery of phosphorus added to dyes

Sample	Na_3PO_4 in dye, %	Na_3PO_4[a] rec., mg	Rec., %
FD&C Blue No. 1	0.012	1.233	102.8
FD&C Blue No. 2	0.302	1.222	101.8
FD&C Green No. 3	0.899	1.203	100.3
FD&C Red No. 2[b]	0.077	1.196	99.7
FD&C Red No. 3	0.000	1.179	98.3
FD&C Red No. 4	0.000	1.209	100.8
FD&C Red No. 40	0.035	1.196	99.7
FD&C Yellow No. 5	0.000	1.183	98.6
FD&C Yellow No. 6	0.000	1.173	97.8
Citrus Red No. 2	0.000	1.248	104.0
Orange B	0.043	1.200	100.0
D&C Blue No. 6	0.000	1.281	106.8
D&C Blue No. 9	0.019	1.222	101.8
D&C Green No. 5	0.154	1.202	100.2
D&C Green No. 6	0.046	1.164	97.0
D&C Green No. 8	0.000	1.189	99.1
D&C Orange No. 4	0.008	1.202	100.2
D&C Orange No. 5	0.342	1.212	101.0
D&C Orange No. 17	0.017	1.210	100.8
D&C Red No. 8	0.000	1.222	101.8
D&C Red No. 10[b]	0.001	1.195	99.6
D&C Red No. 17	0.008	1.230	102.5
D&C Red No. 19	0.010	1.264	105.3
D&C Red No. 21	0.485	1.159	96.6
D&C Red No. 22	0.014	1.185	98.7
D&C Red No. 28	0.045	1.189	99.1
D&C Red No. 30	0.007	1.226	102.2
D&C Red No. 33	0.238	1.182	98.5
D&C Red No. 34	0.021	1.170	97.5
D&C Red No. 36	0.000	1.269	105.8
D&C Red No. 37	0.176	1.243	103.6
D&C Red No. 39	0.007	1.193	99.4
D&C Violet No. 2	0.000	1.216	101.3
D&C Yellow No. 8	0.812	1.165	97.1
D&C Yellow No. 10	0.002	1.253	104.4
D&C Yellow No. 11	0.003	1.220	101.7
Ext. D&C Green No. 1[b]	0.012	1.143	95.3
Ext. D&C Yellow No. 1[b]	0.006	1.264	105.3
Ext. D&C Yellow No. 7	0.035	1.160	96.7
Av.			100.6
Range			95.3–106.8

[a] KH_2PO_4 added equivalent to 1.200 mg (0.300%) Na_3PO_4.
[b] Delisted.

They were asked to analyze the practice samples at least 3 times, and to proceed with analysis of the unknowns only if their results were reasonably close to the stated values.

They were requested to make only single determinations on the unknowns, but to repeat with a smaller sample if the absorbance reading was higher than that of the 2.0 mg standard. If this occurred, they were asked to determine the proper weight by diluting a 25 mL aliquot of the yellow molybdovanadophosphoric acid solution with 25 mL of vanadomolybdic acid reagent, repeating this dilution procedure on the diluted solution if necessary until the absorbance was below that of the 2.0 mg standard. They were requested to report the absorbance of this final dilution, along with the dilution factor, on a separate sheet so the phosphorus content of the 0.4 g sample could be calculated for comparison with the results obtained on the smaller sample.

Table 2. Precision of method with straight dyes

Sample	% Na_3PO_4 Day 1	Day 2	Day 3	Day 4	Day 5
FD&C Green No. 3	0.902	0.895	0.916	0.925	0.911
	0.906	0.898	0.922	0.935	0.915
D&C Yellow No. 8	0.785	0.772	0.802	0.811	0.772
	0.782	0.777	0.796	0.806	0.775
D&C Red No. 21	0.491	0.485	0.499	0.513	0.474
	0.491	0.488	0.497	0.518	0.497
FD&C Blue No. 2	0.301	0.303	0.312	0.314	0.309
	0.299	0.304	0.311	0.314	0.308
D&C Red No. 33	0.233	0.235	0.239	0.239	0.238
	0.232	0.236	0.231	0.233	0.228
D&C Green No. 5	0.146	0.147	0.145	0.143	0.146
	0.146	0.147	0.156	0.142	0.140

Phosphorus

Official First Action

(Applicable to all straight color additives)

34.B07 ***Principle***

Sample is ashed with cellulose powder and MgO. Non-orthophosphates are hydrolyzed to orthophosphates (only form measured) which react with MgO to form heat-stable Mg_3PO_4. Ash is dissolved in vanadomolybdic acid reagent, and phosphate reacts to form yellow molybdovanadophosphoric acid which is measured at 400 nm in 1 cm cells. P content of the sample is expressed as % Na_3PO_4.

34.B08 ***Apparatus***

(a) *Muffle furnace.*—Elec.; with pyrometric control.

Table 3. Effect of re-ashing on percent recovery of phosphorus added to straight dyes

Sample	% Recovery[a] After ashing	After re-ashing
D&C Yellow No. 8	12.9	97.1
D&C Yellow No. 11	72.7	101.7
D&C Red No. 22	88.8	98.7
D&C Orange No. 17	90.7	100.8
D&C Red No. 28	91.0	99.1
Citrus Red No. 2	94.1	104.0

[a] KH_2PO_4 added equivalent to 1.200 mg (0.300%) Na_3PO_4.

(b) *Spectrophotometer.*—Capable of measurements at 400 nm; equipped with matched 1 cm cells.

34.B09 *Reagents*

(a) *Ashing reagent.*—Place 392 g cellulose powder (Whatman CF-11, Whatman, Inc., 9 Bridewell P1, Clifton, NJ 07014) into 4 L erlenmeyer, add 8.00 g MgO, stopper, and mix several minutes by revolving and shaking flask.

(b) *Vanadomolybdic acid reagent.*—Dissolve 20.0 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ in 200 mL hot H_2O. Cool and dil. to 1 L with H_2O. Dissolve 0.500 g NH_4VO_3 in 100 mL hot H_2O, add 100 mL HNO_3, cool, and dil. to 1 L with H_2O. Gradually *add molybdate soln to metavanadate soln* while mixing. Store at room temp. in polyethylene or Pyrex bottle. Reagent is stable several months in Pyrex and longer in polyethylene container. Discard reagent if ppt forms.

(c) *Std solns.*—(*1*) *Stock soln.*—Equiv. to 8.0 mg Na_3PO_4/mL. Dissolve in H_2O, 6.6408 g KH_2PO_4 dried 2 h at 105° (if assay <100% KH_2PO_4, divide 6.6408 g by % KH_2PO_4/100 to obtain corrected wt), and dil. to 1 L with H_2O. (*2*) *Working solns.*—Dil. 0, 5, 10, 15, 20, and 25 mL stock soln to 1 L to obtain equiv. of 0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 mg Na_3PO_4/10 mL, resp.

34.B10 *Preparation of Standard Curve*

Place ca 2 g ashing reagent (beaker pre-calibrated to 2.0 ± 0.3 g is satisfactory and saves time) into sep. 100 mL Pyrex beakers. Add 10.0 mL each working std soln and swirl to mix. Heat at 105° until completely dry (ca 3 h). Cool, and mix with small stirring rod while rotating beaker in tilted position to obtain tumbling action. Place beaker upright and shake gently to level contents while retaining loose condition to expedite ashing. Transfer beaker to cool muffle in ventilation hood (metal tray for beakers facilitates handling) and ash ≥3 h at 500°. (Do not open muffle door before smoking has stopped.) At end of ashing, open muffle door, and let cool ca 1 h before removing beakers. Let beakers cool to room temp.

Add 50.0 mL vanadomolybdic acid reagent, slowly at first until ash is wet, and then rapidly. Swirl and wait several minutes for ash to dissolve. Swirl again and filter thru glass wool (Corning 3950, or equiv.) pledget (filter paper adsorbs color complex) which should fill entire stem of Pyrex powder funnel (65 mm diam. top, 12 mm id stem, 28 mm long). Hold stem end against flat surface and pack glass wool tightly with Pyrex rod ca 15 cm long × 6 mm diam. Collect filtrate in 50 mL g-s erlenmeyer (which can be used to support funnel). Stopper flask and shake to mix. Let color develop ≥10 min; for greatest accuracy, allow approx. same color development time for each soln.

Fill ref. and sample cells with vanadomolybdic acid reagent, and adjust to 0 *A*. Det. *A* for each std soln at 400 nm. Correct readings by subtracting absorbance of 0.0 mg Na_3PO_4/10 mL std (usually 0.005). If *A* of 0.0 mg std is abnormally high, repeat entire detn for this std with ≥3 beakers positioned in different locations in muffle, and use av. value to correct *A* of other stds and samples. Plot *A* vs mg Na_3PO_4/10 mL if desired; otherwise calc. slope of curve as described below. It is not necessary to run stds for each analysis if av. slope for several days gives desired accuracy.

34.B11 *Determination*

Accurately weigh ca 0.4 g dye sample, and transfer to 100 mL Pyrex beaker (free of scratches or other defects) contg ca 2 g ashing reagent. If sample is granular, add ≥10 mL H_2O to completely dissolve, swirl to mix well, and dry completely in 105° oven. Mix and ash as for stds. Develop color and det. *A* of samples in same manner as for stds, allowing ca same color development time for greatest accuracy. If sample *A* is greater than *A* of 2.0 mg std, est. proper wt required and repeat analysis. Correct readings by subtracting absorbance of 0.0 mg std.

Rinse glassware promptly after use with tap and distd H_2O. Soap or detergent should not be used because of possible phosphate contamination.

To expedite analysis, dispense vanadomolybdic acid reagent with pipet or buret with tip cut off; accuracy depends on addn of same, rather than specific, vol. to each beaker.

If yellow crystals form in automatic pipet or buret, remove with dil. NH_4OH soln.

34.B12 *Modifications Required for Certain Dyes*

(*1*) *Ext. D&C Green No. 1:* Filter molybdovanadophosphoric acid soln a second time, using another pledget of glass wool to remove iron oxide.

(*2*) *FD&C Red No. 3:* Add 5 mL HNO_3 (1 + 4) to dye ash and heat on steam bath until dry to remove interfering iodine.

(*3*) *Citrus Red No. 2, D&C Orange No. 17, D&C Red No. 22, D&C Red No. 28, D&C Yellow No. 8, and D&C Yellow No. 11 (also any other dyes with ash*

contg appreciable amt of C): Break up ash with small stirring rod, add ca 2 g cellulose powder, mix, and re-ash 3 h at 500°.

34.B13 ***Calculations***

$$\% Na_3PO_4 = A \times S \times 0.1/W$$

where A = corrected A of sample at 400 nm; S = slope of std curve = r/n; r = sum of ratios of mg Na_3PO_4/10 mL to A of each resp. std; n = no. of std solns used in calcn; 0.1 = 100/1000 (nos. used to convert to percent and mg to g, resp.); W = g sample.

Results and Discussion

The results obtained by the collaborators, along with statistical data, are shown in Table 4. The repeatability was very good, as indicated by within-laboratory coefficients of variation (CVs) ranging from 1.67 to 4.66%. The reproducibility was good, as shown by between-laboratory CVs which ranged from 2.59 to 5.54% with the exception of Sample 1, which was 8.79%. The single determination CVs were satisfactory, ranging from 3.76 to 7.39% with the exception of Sample 1, which was 9.60%.

Table 4. Collaborative results by the spectrophotometric molybdovanadophosphate method for phosphorus in certifiable straight color additives, expressed as percent trisodium phosphate

	Sample[a]					
Coll.	1	2	3	4	5	6
A	6.59	2.40	0.313	0.567	0.257[b]	0.714
	6.61	2.38	0.317	0.564	0.283[b]	0.712
B	7.61	2.38	0.361	0.541	0.371	0.742
	7.50	2.67	0.343	0.558	0.370	0.775
C	7.65	2.51	0.308	0.525	0.334	0.729
	7.71	2.34	0.301	0.553	0.332	0.746
D	6.31	2.30	0.335	0.566	0.346	0.677
	6.33	2.29	0.324	0.576	0.324	0.692
E	4.87[b]	2.28	0.322	0.496	0.332	0.690
	4.48[b]	2.28	0.307	0.503	0.322	0.701
F	6.72	2.39	0.326	0.588	0.344	0.714
	6.93	2.34	0.287	0.523	0.334	0.699
G	5.73	2.12	0.340	0.553	0.346	0.739
	6.61	2.35	0.372	0.547	0.364	0.730
Av.	6.86	2.36	0.325	0.547	0.343	0.719
Within-lab.						
SD	0.26	0.11	0.015	0.020	0.009	0.012
CV, %	3.85	4.66	4.615	3.656	2.624	1.669
Between labs.						
SD	0.60	0.06	0.018	0.019	0.015	0.025
CV, %	8.79	2.59	5.538	3.473	4.373	3.477
Single detn						
SD	0.66	0.13	0.024	0.027	0.018	0.027
CV, %	9.60	5.34	7.385	4.936	5.248	3.755

[a] 1 = D&C Orange No. 5; 2 = D&C Yellow No. 8; 3 = FD&C Blue No. 2; 4 = FD&C Red No. 3; 5 = FD&C Red No. 40; 6 = FD&C Green No. 3.
[b] Rejected as statistical outliers.

The following change was made in the method after the collaborative study: Under "Determination," lines 7 through 10 to now read: "If sample absorbance is greater than absorbance of the 2.0 mg standard, dilute an aliquot of the yellow molybdovanadophosphoric acid solution with vanadomolybdic acid reagent until the color intensity is lower than that of the 2.0 mg standard. Read absorbance, and correct percent Na_3PO_4 for dilution." The collaborators' results from testing this procedure showed that dilution gave erroneously low values, apparently because of deviation from Beer's law, so it was revised to read: "If sample absorbance is greater than absorbance of 2.0 mg standard, estimate proper weight required and repeat analysis." Collaborators' results obtained with the defective dilution procedure were not used in Table 4. Rather, the results used were the corresponding results which they obtained on a smaller sample whenever the initial 0.4 g sample gave absorbance readings higher than those of the 2.0 mg standard.

In summary, the method is simple, rapid, and precise, and as measured by recovery studies, gives accurate values for total phosphorus. It is applicable to all certifiable straight color additives.

Recommendations

It is recommended that the proposed spectrophotometric molybdovanadophosphate method be adopted official first action for the determination of phosphorus in certifiable straight color additives, and that study of the topic be continued.

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REVIEW OF ANALYTICAL METHODS FOR SULFONAMIDES

Analytical Methods for Sulfonamides in Foods and Feeds. II. Performance Characteristics of Sulfonamide Methods

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Important factors in interpretation of methods for sulfonamides in tissues are value of the blank, use or omission of recovery factors, and precision of the methods. For determining sulfonamide in tissues, no interlaboratory collaborative studies have been performed to provide reproducibility parameters. By assuming comparability with other tissue residue methods at equivalent concentrations, it may be anticipated that the coefficient of variation *within*-laboratories of the Bratton-Marshall method is about 15% at concentrations of a fraction of a part per million. It is estimated that the limit of reliable measurement of the Bratton-Marshall method is about 0.2 ppm, varying with the individual laboratory. This value is higher than the tolerance it is intended to enforce. Obviously, the method in this case has been stretched beyond its original claimed capabilities. This method also has high blanks and low recoveries. Assignment of sufficient resources to the solution of the problem by regulatory agencies has resulted in methods capable of handling the sulfonamide residue problem at 0.1 ppm.

Associated with methods of analysis are a number of performance characteristics or attributes which permit them to be evaluated and compared. These performance characteristics fall into 2 general categories: *technical or scientific*, which answer the question whether the measurements obtained will provide interpretable information; and *administrative or practical*, which determine if the information will be obtained within a reasonable time and at a reasonable cost.

Attributes of the first category provide information about the reliability of a method; those of the second category provide information about its practicability. These attributes are discussed in general in the recent book by Massart et al. (1) and in the chapter on analytical food chemistry by Horwitz (2).

These general discussions treat attributes as if they are fundamental constants of analytical methods, which they are not. Attributes are properties of methods which, when measured in different laboratories at different times under different circumstances, provide different answers. Most frequently the results obtained by users of methods reflect considerably poorer performance than those initially reported by the developers of methods. As Banes (3) pointed out, the creators of new analytical methods are rarely unbiased reporters. The frequency with which methods submitted in support of petitions for food additive regulations fail to be validated when tested in Food and Drug Administration (FDA) laboratories attests that methods are sensitive to laboratory environment and analyst inexperience. Since the results of even successful validation trials are not published, there is a vacuum in our knowledge of the true performance of methods under practical laboratory conditions. Only if a collaborative study of a method has been performed under conditions specified by the AOAC and similar organizations is enough initial information available to arrive at a decision on the probable performance of an analytical method under the best conditions. Performance under practical conditions can then be monitored by participation in check sample series such as those conducted by the American Association of Feed Control Officials (AAFCO).

AOAC official methods for sulfonamides (SF) are available only for several specific compounds added to feeds and premixes at levels of several hundred parts per million. No collaborative studies have been performed with methods applicable to tissue residue levels. Therefore, much of the discussion of the performance characteristics of the methods used to monitor and regulate SF levels in tissues is based upon intuition and experience with similar methods which have been collaboratively studied at approximately the concentration levels of interest.

Accuracy

Strictly defined, an accurate method is one that is free from bias, or that gives the "correct" result. However, it is impossible to know the "true"

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value in the case of tissue residues, because the real concentration desired is that of the native, incurred, or transformed residue deposited by the animal. There are 2 practical approaches to determining the accuracy of residue methods under such conditions—the methods of additions and of exhaustive extraction. Both of these methods assume that their extraction steps liberate all of the bound residue of interest.

Method of additions.—The accuracy of a tissue residue method is often measured in terms of recovery of SF added to a tissue sample. Known amounts of SF are added to control (negative) tissues to provide "spiked" samples. The spiked tissues must be held long enough for any interaction with tissue components to attain equilibrium. If some analyte is present in the tissue used as the base for spiking, the amount present can be estimated by the method of additions. In this technique, several different concentration levels are added to portions of the same well-mixed tissue and the amounts found are plotted against the amounts added. The line of best fit extrapolated back to the x axis provides an estimate of the amount of analyte present in the unfortified tissue. The individual additions corrected for the amount present initially provide an estimate of the recovery by the method.

Method of exhaustive extraction.—In this method the substrate is extracted until additional portions fail to remove any more residue. The procedure assumes that a reversible equilibrium exists between the factors retaining the residue and the solvent removing it. It will not handle mechanical problems such as channeling or failure of the solvent to penetrate the tissue, or chemical problems such as the binding of residues to tissue components.

Both methods, additions and exhaustive extraction, will fail to measure bound residues that are not extracted by the method. The problem of metabolites complicates the definition of accuracy by requiring a definition of what is to be measured—the parent compound added, the parent compound incurred in the residue, or the total residue related to the administered or added drug (parent plus metabolites)—and, if metabolites are to be included, how far down (or across) in the metabolic chain are transformed materials to be encompassed?

Because such questions are very difficult to answer, accuracy is usually defined in residue studies as the recovery of the parent compound and/or major metabolite(s) recovered in spiked recovery studies.

Colorimetric Methods

Most Bratton-Marshall (B-M) types of methods (4) claim 90–100% recoveries of spiked samples at the critical tolerance level of 0.1 ppm. In many cases, however, it is not clear how this result was achieved, in view of the complicating effect of the background blank and recovery factors.

Most methods compensate for background pigments or turbidity by using a background correction (or non-*N*-naphthylethylenediamine dihydrochloride) blank in the reference cell or by measuring it directly and subtracting its value from that of the sample value. This background blank is lower than a control blank (from presumably SF-free tissue) but of the same order of magnitude.

Control blanks.—The blank from control tissues is responsible for much of the inaccuracy of the SF tissue assay at the tolerance level for 2 important reasons:

(*1*) It is impossible to know the absolute value of the control blank associated with any given tissue because current cleanup procedures do not remove all chromogenic material. Furthermore, this blank is not reproducible from assay to assay. Because the compounds responsible for the control blank have not been identified, it has been impossible to systematically develop a procedure to eliminate them, if in fact they are non-SF compounds. Because the sample being analyzed does give a measured value, there is no parent zero control tissue that corresponds to this sample. The control tissue is actually tissue from animals assumed to have been raised under SF-free conditions and supposedly free from SF. The validity of this assumption is questionable in view of the widespread low-level contamination found in nonmedicated feeds and control tissues. The important point is that no true blank is available to correct individual values for an imperfect cleanup. As a practical matter, an average historical blank for a particular tissue is often assumed and applied as a correction factor in recovery experiments and sample measurements.

(*2*) The assumed blank is present at a level that constitutes an appreciable fraction of the tolerance, a condition which does not correspond to good analytical practice. At the tolerance level of 0.1 ppm and a blank value of 0.05 ppm, the blank constitutes 33 or 50% of the measured absorbance, depending on how the calculation is made (using 0.10 or 0.15 in the denominator). By either method of calculation, the value exceeds

a reasonable blank of ≤10% of the total measurement.

Recovery factors.—The second important point affecting the accuracy of B-M types of methods is the use of recovery factors. A recovery of 90–100% of SF added to tissues is usually reported in research papers. In routine analysis, the recoveries are considerably lower, possibly because of different equilibrium conditions or methods of calculation. Recoveries as low as 80% can still be considered satisfactory, but recent carefully conducted recovery studies using the Tishler methods (4) by Food Safety and Quality Service (FSQS) and FDA laboratories showed recoveries of only about 50% at the 0.1–0.5 ppm levels. Such values apparently are typical of routine laboratory work in SF residue analyses. A possible explanation is that many of the literature methods may include an undisclosed correction factor that is routinely applied in the reported data. Such correction factors are applied automatically by FSQS in their monitoring program. Correction factors are not applied routinely by FDA laboratories. FDA reports the calculated uncorrected values and the recoveries separately, preferring to use the correction factor as an error allowance before concluding that an over-tolerance residue is present. Only the sulfanitran method of the *FDA Food Additives Analytical Manual* (5) specifically mentions correction of observed values. Several of the other methods in the manual refer to recoveries and spiked samples, but do not give explicit directions for using a recovery factor.

Applying correction factors and reporting the corrected result increase the variance of the corrected value, because 2 error terms are included in the reported value. By using correction factors, error from a constant laboratory bias is eliminated, thus discouraging the regulated industry from shopping for the laboratory with the lowest recovery factor. On the other hand, application of correction factors hides the recovery variability and eliminates the possibility that laboratory results will be rejected on the grounds of too low a recovery. When recovery factors were used for correction, quality control, or certification, the question arises as to which of the several recovery factors are to be used: the factor associated with tissues conducted along with the samples, an average value typical of that laboratory, a moving average over a definite time period, a constant pooled value from many laboratories, and various other possibilities, including weighting factors. There is also a legal aspect: The tolerance is set on the basis of toxicological response but monitored on the basis of independent chemical observations that would not correspond to the scale used in setting the tolerance, if the results are not corrected to 100% recovery.

Feed methods.—Accuracy of the collaboratively studied AOAC methods for SF in feeds is satisfactory for regulatory purposes (Table 2 in ref. 4), with the exception of sulfadimethoxine. The assay for this SF, as discussed in the section on statistical parameters, has more significant problems with precision.

Chromatographic Methods

With chromatographic methods—thin layer (TLC), liquid (LC), and gas (GLC)—the extraction problem still exists, but the blank problem becomes fundamentally different. Even if the same cleanup were used as for colorimetric methods, chromatographic procedures should handle the background materials differently in the determinative steps. Yet the literature still reports an SF blank with these chromatographic methods on control tissues. SF seem to be an unlikely laboratory contaminant; therefore, the more likely explanation is a ubiquitous contamination from SF in animal production, either from feed or environment.

Chromatographic methods have inherently lower absolute recovery factors because of column or adsorbent losses. Therefore, absolute recoveries as low as 60% are not unusual, although a significant fraction of the loss may be attributable to the cleanup partitioning steps. This defect can be corrected to an appreciable extent by conducting the standards through the entire procedure, but the correction is not entirely satisfactory because standards may behave differently in the presence of the normal substrates than when they are present without the substrate. If the calibration curve is prepared with control tissues, the blank correction enters into the determination twice—once in the calibration curve and once in the actual determination. Such a standard curve has a greater variability than the common standard curve prepared from the pure standard at the final determinative step of the method.

Precision

Precision (or imprecision) is a measure of the variability of values around a mean, measured as a standard deviation (SD) or coefficient of variation (CV). The precision can consist of a number of components, depending on the experimental design of the system used to estimate it.

Table 1. Statistical parameters for residues of feed additives in tissues from AOAC collaborative studies

Compound	Method, 13th Ed., 1980 (JAOAC reference)	Determinative step	Concn, ppm	Interlab. CV, %	Spiked rec., %	Tissue
Clopidol	**41.013–41.018** (**57**, 914 (1974))	GLC		7	84	muscle
				5	90	liver
				14	84	eggs
			0.15	15		muscle
			0.15	22		liver
			0.15	18		eggs
Decoquinate	**41.019–41.023** (**56**, 71 (1974))	LC fluorometric	0.1	13	100	muscle
			0.3, 1.0	7	100	muscle
			0.1	21	120	liver
			1	9	95	liver
			2	11	100	liver
Nalidixic acid	**41.036–41.039** (**53**, 464 (1970))	LC fluorometric	0.1	24	93	muscle
			0.1	30	95	liver
Ethoxyquin	**41.024–41.028** (**51**, 537 (1968))	fluorometric	0.4	10	80	eggs, muscle
			2	8	85	liver, fat
Zoalene	**41.040–41.046** (**49**, 708 (1966))	LC spectrophotometric	1.5	11	75	muscle
ANOT	**41.001–41.008** (**49**, 708 (1966))	LC B-M	1.5	9	80	muscle
Melengestrol acetate	**41.029–41.035** (**59**, 507 (1976))	chromatographic cleanup	0.01	16	98	fat
		GLC	0.02			

The most important aspect of precision reflects variability between laboratories, often termed *reproducibility;* another important component is the within-laboratory variability, often termed *repeatability.* Most publications give only the repeatability term, which consists primarily of the variability of the analyst. Reproducibility determinations require considerable organizational effort like that of the AOAC. However, it is reproducibility that is of greatest importance to FDA and FSQS, because they must be able to check each other, state laboratories, and commercial laboratories who may examine any violative sample at the request of a claimant or defendant. As noted above, no collaborative studies have been performed on methods for determining SF residues in tissues. The few so-called validation studies that have been made, consisting of analyses by experienced analysts on identical samples in no more than 2 laboratories, are practically useless for estimating between-laboratory statistical parameters.

We can approximate the precision of SF residue methods on the basis of 2 assumptions: (*1*) The overall interlaboratory precision of a method is, in general, a function of the concentration of the analyte (rather than a function of the nature of the method or of the analyte) (p. 602 in ref. 2); and (*2*) the SF methods will perform much like current residue methods for which data on interlaboratory performance do exist.

Methods for the determination of 7 compounds as residues in animal tissues have been collaboratively studied under AOAC auspices. The data are summarized in Table 1. The concentration range is 0.01–2 ppm in a wide variety of animal tissues (muscle, liver, eggs, fat). The average recovery from spiked tissues is about 90% and the pooled interlaboratory CV is about 14%. High and low CVs do not appear to correlate with the nature of the tissue, nature and level of analyte, or type of determinative step. Interlaboratory studies of methods for the determination of pesticide residues at about the same concentrations (6) also show a mean recovery of about 95% and an interlaboratory CV of 15%.

In extrapolating these data to practical laboratory operating conditions, it must be remembered that interlaboratory data were obtained under the best possible conditions. Although the laboratories did not know the actual concentrations of the analyte in the samples, they did know that the samples were not routine and presumably used their best analyst who, know-

ing this was a test, worked carefully. Therefore, reproducibility and repeatability under practical working conditions, where less attention may be given to each sample, will probably be poorer than under test conditions. Comparison of the CV of various methods reported in the literature with the permitted analytical variations of the FDA and AAFCO shows that CV increases under practical operating conditions. However, CV decreases to a constant value with analyst experience (7).

Experience with methods has shown that the within-laboratory variability is 1/2 to 2/3 the total variability. Therefore, by extrapolation of literature within-laboratory data, analyses by the colorimetric B-M method should have a between-laboratory CV of about 15%, if all the laboratories used the same procedure. Recent inspectional findings, however, indicate that laboratories are introducing their own modifications of the published directions. This may be a neglected aspect of good laboratory practices and tends to defeat the purpose of good analytical chemistry.

It must be emphasized that this estimate is made because of the lack of data; there is no substitute for an interlaboratory study on the final method selected for use in regulatory and monitoring programs.

A further complicating factor, often overlooked in evaluating the reliability of SF methods, is the necessity to replicate the control blank as well as the measurements, because the blank value must be subtracted from the sample value. Although this is considered a precision factor because it is reduced by replication, the variability of the control blank affects both accuracy and limit of reliable measurement. The variability (S_D^2) of the net difference of the final reported value is a function of the pooled variance of the sample measurement and the control blank measurements. The individual variances are reduced by replication when they enter the difference as follows:

(S_D^2)	$= S_S^2 + S_B^2$	
(Std error of sample)^2	$= S_S^2$	= (SD of sample)^2/No. of replications
(Std error of blank)^2	$= S_B^2$	= (SD of blank)^2/No. of replications

It is seen from the first equation that if the sample measurement is replicated but the blank is not, a greater percentage of the blank variability than sample variability will be included in the final net variability of the difference. To illustrate, if we have a measurement of SF of 0.15 ppm with an SD of 0.02, and a blank of 0.05 with an SD of 0.01, the standard *error* of the difference ($\sqrt{S_D^2}$) as a function of the number of replications is as follows:

No. sample measurements (*SD = 0.02*)	*No. blank measurements* (*SD = 0.01*)	*Std error of diff.*
1	1	0.022
16	1	0.011
1	16	0.020
16	16	0.006

This tabulation shows the necessity for replicating both the blank and the measurement when both are variable in order to minimize the variability of their difference.

Equally instructive is the outcome when different combinations of replications are considered. Consider the same statistical parameters, but with 4 and 2 replications instead of 16:

No. sample measurements (*SD = 0.02*)	*No. blank measurements* (*SD = 0.01*)	*Std error of diff.*
1	1	0.022
4	1	0.014
1	4	0.021
4	4	0.011
2	2	0.016
2	1	0.017
1	2	0.021

To improve precision it is most profitable to make the most measurements of the factor having the most variable component (in absolute terms).

Specificity

Specificity of a method is the characteristic that responds exclusively to the constituent being sought. To be specific, a method must exclude all other compounds that respond to the determinative step through separations (cleanup), or, if they are not separated, through specific detectors. Specificity must also exclude nondescript response from general background arising from the matrix (sample) or from the environment.

In residue analysis, we must ask whether the tolerance applies to the specific compound or to the group of compounds with similar pharmacological actions. Tolerances are usually listed in the appropriate sections of the *Code of Federal Regulations* (CFR) for the individual compounds.

For SF, the tolerances, 0, 0.01, or 0.1 ppm, apply to a specific SF in the individual, edible, uncooked tissue of a specific animal. However, 21 CFR 570.18, "Tolerances for related food additives," requires food additives that cause similar or related pharmacological effects to be considered as a class. When declaring all SF-containing drugs to be new drugs because of their action on the thyroid, the Commissioner of Food and Drugs did consider all SF drugs as a distinct pharmacological class (21 CFR 510.450). When 2 or more chemicals of the same class are present, the tolerance for the total is the same as that for the additive with the lowest tolerance, unless the individual amount of each additive present can be determined quantitatively, in which case the combined tolerance can be calculated as the weighted average (in proportion to the amounts present) of the individual tolerances.

Since there are actually 2 tolerances for SF in tissues (the 0.01 ppm is applicable only to sulfadimethoxine in milk), the specificity problem for SF in tissues is to determine if the SF found is in the class with the zero tolerance or the class with the 0.1 ppm tolerance. This problem exists only when the amount of residue is below 0.1 ppm, where the quantitation admittedly is unreliable. If the amount of residue is above 0.1 ppm, the specificity problem is to confirm the identity of the SF moiety, not necessarily the individual compounds. Sometimes, however, it may be easier to confirm the identity of the individual compounds than to confirm the presence of the SF group.

The nature of the material to be identified affects the nature of the residue chemistry required. If total SF are to be determined, specificity for individual compounds is superfluous and a selective cleanup for SF as a class followed by determination with a nonspecific reagent such as the B-M is the appropriate method. Combinations of detectors responsive to sulfur and nitrogen atoms, or even the characteristic polarographic wave of SF, may be adequate.

If confirmation of the identity of the individual compounds is required, the preferred current technique is isolation of SF from tissue components (cleanup), preliminary identification by GLC or TLC, and confirmation of identity by mass spectrometry (MS). Although 10 years ago the use of MS would have been considered impractical, the advent of lower priced and simpler instruments is making MS a more practical analytical technique. However, interpretation of spectra requires experience, and special MS techniques are needed to differentiate the isomeric SF diazines (pyridazine, pyrimidine, and pyrazine).

Simpler confirmation techniques, using less esoteric and less expensive equipment, may be developed by using different solvent systems with TLC and different column polarities with GLC, with interpretation through the application of information theory. An elementary discussion of this technique is given in Chapter 8 of the book by Massart et al. (1). Calculations can be made of the relative efficiencies of various systems and their combinations in terms of resolving power of the chromatographic techniques and the spectral peaks of MS.

A general comparison of the relative specificity of the various techniques for identifying the SF group and the individual SF is as follows:

Technique	*SF group*	*Individual compounds*
Tishler method	fair	not applicable
TLC	poor	good
TLC + B-M applied to spots	good	good
GLC	not applicable	good
GLC + derivative	not applicable	very good
LC	not applicable	good
MS	good	excellent

The combination of several good techniques, derivatization, and multiple polarity systems improves the certainty of identification.

Limit of Reliable Measurement

The limit of reliable measurement (given as *lower* limit of reliable measurement in 21 CFR 500.90(d)(5)(reproposed)) (8) appears to be a simple concept until it is examined in detail. It means the lowest amount (or concentration) of a substance that provides a "reliable" measurable response. We are ordinarily not interested in the upper limit; it is easily bypassed by aliquoting, diluting, or taking a smaller sample size. The problem now becomes one of defining a reliable measurable response. The measurement is almost always taken against a background of the blank or of the "noise" of the measuring instrument. The blank has a distribution characterized by a mean and SD. The analyte also yields a signal which has a distribution with a mean and SD. The question now becomes, "When do the 2 means differ significantly from each other?"

which is a conventional statistical specification problem. The analyst must assign a degree of confidence to his desire for being correct or a probability that he is willing to assume of being wrong; then the SD of the 2 distributions (signal and noise or blank) will permit a calculation of the limit of reliable measurement (Chapter 6 in ref. 1).

When formulated in this manner it becomes obvious that insufficient information is available in the literature to assign a limit of reliable measurement to any of the SF residue procedures. Various statements are given with literature methods that the procedure is capable of detecting or determining certain minimum amounts of SF. But such statements are not accompanied by the SD of the measurement or values for the corresponding mean and SD of the blank or noise.

For example, consider that the quantitative SF procedure gives a mean of 0.1 ppm on a certain sample and SD of 0.02 ppm, and there is no blank or noise. What value is the lower limit of reliable measurement? If we decide that we will tolerate only a 1% chance of being wrong, the answer is 0.15 ppm $((2.58 \times 0.02) + 0.10)$ for a single measurement. The value 2.58 is the statistical multiplier associated with the choice of 1% probability. If we must consider a blank with its mean and distribution, the calculation obviously becomes more complicated because we must consider the intersection of 2 distributions. In addition, we must remember that all of these probability distributions are based on the application of the normal distribution; yet measurements near the limit of reliable measurement are probably not distributed normally. With all of these assumptions, it is likely that the limit of reliable measurement of the B-M (Tishler) method is $\geq$0.2 ppm.

Usually when statistical calculations are made, the raw data are given to a statistician who may not have any conception of the physical realities involved in obtaining the data and using the results. He analyzes the data according to standard statistical techniques and draws conclusions of statistical significance or nonsignificance. The chemist who receives this information may not be familiar with the assumptions involved and the statistical vocabulary; thus he proceeds to act on the values given to him with a certainty that might not be warranted if he were fully aware of the inherent qualifications and assumptions hidden in the calculations.

As an example of what can be done with a single set of data, Currie (9) listed 8 definitions of the limit of detection (not the same as, but related to, the limit of reliable measurement). These definitions involved the following types of data: the background SD, 10% of the background, 2 or 3 times the SD of the background, the sum of 3 times the SD of the background plus 3 times the SD of the sample, twice the background, and several other arbitrarily chosen background values. Depending on which assumption is taken, the limit of detection for the particular measurement used as the example varied from about 5 to 3000 picocuries! Neither FDA nor FSQS has given consideration to reasonable assumptions to use in calculating a limit of reliable measurement for SF.

To complicate the matter further, for simplicity the limit of reliable measurement is taken as an absolute, unvarying characteristic of an analytical method. Unfortunately it is not; rather, it varies within a laboratory according to analyst, instrument, sample, and weather conditions. An even greater variability in this attribute is introduced when several laboratories are involved.

In regulatory work, the variability of greatest importance is that between laboratories, since government laboratories must check themselves as well as the manufacturer. As mentioned previously, the between-laboratory SD is approximately twice that of the within-laboratory SD in many cases. Such a variability would require an action level for SF based on a limit of reliable measurement even greater than the 0.2 ppm arrived at from within-laboratory values for the Tishler method. Thus, the limit of reliable measurement for the Tishler method is above the tolerance which has been set for SF in animal tissues. There are not enough data to estimate the limit of reliable measurement of the new chromatographic methods: TLC, GLC, and HPLC.

Sensitivity

The term sensitivity has been used in analytical chemistry in at least 2 different senses, making it necessary to define the term whenever it is used. Very often it is used as the limit of detection. However, a more consistent usage throughout science is as the slope of the response curve—the change in response per unit stimulus. Calibration curves are ordinarily used only where the sensitivity is constant. The points where the response curve begins to deviate from linearity at both ends characterize the limits of applicability of the procedure.

The calibration curve is related to the limit of reliable measurement by formulating the prob-

lem in terms of the difference between the response from a sample and from a blank. When the question is phrased in this manner, the answer depends entirely on the definition of significance, which will be a function of how much the distribution of the blank response is permitted to overlap the distribution of the sample response. To be useful, enough data must be available to construct the distribution of both the sample and blank responses. With SF, the experimental designs used in the development of the methods have not been formulated to obtain this type of information. About the best that can be said is that, in general, an absorbance of about 0.1 is obtained for a concentration of 0.1 ppm in the B-M reaction, using the directions of the Tishler modification. In a sense, this is a characteristic of the method. A calibration curve deviating considerably from this value should be rejected.

Outliers

Outliers are those aberrant values that occur even in the best-conducted experiments. They do not lie within the main body of the normal distribution. They are also unexplainable, because if they could be explained as incorrect calculations, improper preparation of the standard or standard curve, or loss of a portion of the sample, they could be rejected or corrected on the basis of the explanation. As a result of examining the collaborative study data for numerous methods, a criterion that $\leq$10% of the data should be outliers in an acceptable residue method appears to be reasonable. It may well be argued that this is a rather high level for regulatory work, but the data from the reported collaborative studies clearly show that aberrant values are inevitable in practical laboratory work at the trace levels involved. Control of outliers is a function of proper supervision and quality control to keep them within reasonable bounds in order to avoid repetition of expensive critical analyses.

The Tishler method for SF, and in fact any of the methods using the B-M reaction, seems to produce an unreasonable number of outliers in the course of ordinary laboratory work. Although this type of data obviously is not being published, this conclusion can be drawn from conversations with those conducting such analyses. Part of the difficulty may lie in the method description as well as in the liberties analysts take with the written version. Although similar information is lacking for the chromatographic methods, they do not appear to produce outliers as frequently as the colorimetric methods, presumably because they contain a degree of specificity in the final measurement which permits rejection of non-SF response so that the values do not become false positives.

The criterion of "dependability" of the proposed "Criteria and Procedures for Evaluating Assays for Carcinogenic Residues" (8) may have been intended to apply to outliers, but both the preamble and the text of the regulation restrict consideration to unfinished assays performed during the construction of calibration curves from spiked tissues.

Practicability

A practicable assay is one that can be performed readily in a regulatory laboratory. It is meant to differentiate this type of an assay from those requiring exotic or expensive equipment found only in research laboratories. In this sense, all of the SF assays discussed here are practicable. They consist of well-known laboratory operations using commercially available reagents and equipment. A number of samples can be completed within 1 or 2 working days, although several days will be required if several techniques are applied consecutively to the same sample. Some of the extractions use solvents that are now considered hazardous, so a hood is required. Diazomethane, required for derivatization before GLC in some procedures, requires special handling for safety. Naturally, laboratories would like faster, cheaper, and more reliable tests, but in general, all of the assays proposed cannot be considered impractical from an operational point of view.

Special Problems

Considering the Tishler method, the only method regarded by regulatory agencies as having sufficient validation for regulatory use, SF methodology suffers from 2 major defects: (*1*) The limit of reliable measurement is higher than the tolerance the method is intended to enforce; and (*2*) the method has a high and variable blank of unknown nature. Neither the Tishler method, nor any other method, quantitative or qualitative, has been validated by an adequate interlaboratory study so that the analytical chemist is able to report a value with full confidence in the identity of the material and the reliability of the amount.

Needed Research

It is difficult to understand the low priority given to research on methods for drug residues

in animal tissues for the past 20 years. Approximately 30% of the food consumed in the United States is of animal origin and almost all animals used for food are exposed to either therapeutic or prophylactic drug treatment at some period during their lifetimes. The widespread administration of drugs to animals used for food has provoked a number of important controversies such as the carcinogenicity of diethylstilbestrol and the public health significance of transfer of antibiotic resistance. Yet these disputes have not stimulated much research on the analytical methods needed to provide the basic information about residues of drugs in tissues from which reasonable safe tolerances could be set. Diethylstilbestrol and SF have competed for the available resources to the detriment of both. Very little attention has been given to the development of specific methods, qualitative or quantitative, for the antibiotics; no program exists to develop a multiresidue method for animal drugs similar to the successful multiresidue methodology for pesticide residues.

Some effort has been made or sponsored by the animal drug industry and the government to provide more useful methods for SF residues in animal tissues, either to support New Drug Applications or to develop more accurate, precise, sensitive, and specific methods for certain SF residues. Although it is almost impossible to optimize all attributes of an analytical method simultaneously (10), particularly at the levels approaching the limit of reliable measurement, little emphasis has been placed on identifying the most important method attributes for controlling the public health aspects of drug residues.

This review has shown that current "approved" methods for SF in tissue, particularly the Tishler method used by FSQS and FDA, lack accuracy (poor recoveries) and precision (high variabilities); no claim has ever been made for specificity. These methods, based on a diazotization reaction, have the defects mentioned above: The control blank is high, and the practical limit of reliable measurement is above the tolerance they are intended to enforce. Yet methods derived from the original B-M procedure are sound. They do not deserve the criticism that has been heaped on them because their performance has been stretched far beyond the original claimed capabilities. The basic B-M method has served as the control for the therapeutic, clinical administration of SF at the 1 ppm level and above. Its diazotization principle has been successfully applied to many families of compounds—active ingredients, impurities, and contaminants—in numerous substrates. It is therefore more profitable to examine the present situation to determine which of these problems can be solved than to seek in vain for the ultimate in methods.

The first question should be: Is 0.1 ppm the proper tolerance for all edible animal tissues? This is a toxicological question, but not beyond the scope of this review, because it determines the goal the analyst must strive to achieve. Historically this tolerance was established, in the absence of long-term animal trials, at what was then considered the limit of reliable measurement for SF, 0.1 ppm. But we now see that 0.1 ppm was a liberal, even exaggerated estimate of the residue chemist's technical skill.

Therefore, the most important question should be rephrased: What is the proper tolerance for SF in animal tissues? If the proper tolerance ought to be 1.0 or even 0.5 ppm, the analytical problem disappears; if 0.1 is too high, the need to improve the methods is even more urgent. In the absence of any new data to support a revised tolerance, no revision has been recommended. What should be initially a toxicological problem has been left by default with the analyst.

The second question should be: Is it necessary to maintain the same tolerance for all edible tissues—muscle, liver, and kidney? If SF are evenly distributed throughout all tissues, the relative consumption pattern of these 3 might justify a different tolerance. Liver and kidney usually show a higher tissue blank than muscle; therefore raising the tolerance for the organ meats would relieve the analytical problem to some extent.

Because of deficiencies in our toxicological knowledge, it is easier to perpetuate a tolerance than to justify a revision. Therefore, the chemist will probably need a method that will reliably determine $\leq$0.1 ppm. No such method is available, however, because of the manifold interferences related to the presence of SF in tissues.

Therefore, in order to provide a reasonable chance of developing a method capable of monitoring 0.1 ppm of the pertinent SF in tissues routinely, accurately, and reproducibly, the problem must be simplified to manageable proportions. FSQS reports that practically all of the SF residues encountered are sulfamethazine (SM) in hogs and sulfaquinoxaline (SQ) in poultry. If we can narrow the requirement to a method for the determination of SM in hog tissue and SQ in poultry tissue, with an ability to indicate the

presence of any other interfering compound (by an extra spot, an extra or distorted peak, an off-color, etc.), we have a much greater chance of success.

Because one of the major problems with current methods is the high tissue blank, better cleanup is essential. In fact, if adequate cleanup can be attained, practically any measurement system will be adequate for the determination. If we need determine only SM, a partition-type cleanup, such as the Tishler, using the specific acidic dissociation constant for SM (K_a), might be adequate to handle nearly all of the samples found positive by FSQS.

The second major difficulty with SF methodology is low recoveries. All of the methods require several steps, with losses inherent at each step. For example, if each of 3 steps entails a 10% loss, the recovery for the entire method cannot exceed 73%. Poor quantitative technique could increase losses still further. The recent use of radioactive tracers should be applied to SF methodology to determine where the losses occur and thereby permit action to be taken to minimize them.

Most statements of requirements for SF methodology include specificity in terms of separation, identification, and confirmation of the individual SF, always in the presence of each other. In practice, this is unrealistic. As discussed previously, the safety problem applies to SF in general, not to the individual SF. Therefore, the specificity desired is directed to the SF moiety. Considerable energy is being misapplied in attempting to identify individual SF simultaneously with quantitation. Methodology can be simplified if only SM needs to be determined initially, and if identification and quantitation of additional SF are not required unless the initial screen for SM indicates the need for additional work. Commercially formulated therapeutic SF contain at most 4 SF compounds including SQ, which is used only with poultry.

Many statements of requirements for SF methodology also include the determination of SF metabolites, the most common of which is the acetyl derivative. Available information indicates that in most cases (tissues and excreta) the parent SF and its acetyl derivative account for approximately 90% of the ingested drug, with the unchanged drug predominating. The SF or derivatives may be conjugated as sulfates or glucuronates. Releasing SF from these derivatives requires a hydrolysis step, which might be accomplished as part of the extraction or which may be unnecessary; but as a first approximation, metabolites, particularly minor metabolites (e.g., ring hydroxylation products), should be ignored. If later research shows that they must be considered in the quantitation of SF in tissues, the necessary steps can be introduced to account for them.

Conclusions

Most research on the development of methods of analysis starts with a requirement for the attainment of the most perfect method imaginable, one that is accurate, precise, specific, sensitive, fast, and cheap. As work proceeds, necessary compromises and trade-offs are made and sights are lowered. In this case we are suggesting a reverse program, i.e., start with simplifying assumptions and introduce the complications after success is achieved.

Current methodology is inadequate to enforce the tolerance of 0.1 ppm residues in *edible tissues.*

Adequate methods are available to monitor prophylactic and therapeutic levels of SF *in feeds,* except for sulfadimethoxine. The corresponding methods for SF in feeds at contamination levels (1–10 ppm) are not satisfactory.

The B-M types of methods are inadequate to monitor a tolerance of 0.1 ppm of SF residue in tissues. A better method is required. The proposed assumptions for the development of a method for SF in tissues are as follows: (*1*) Because the major problem is the presence of SM and the tissue in which it is most likely to be found is liver, confine the problem to the determination of SM in liver; (2) Because the species where the major problem occurs is swine, limit the animal of interest to swine; (3) Because the major residue is SM, limit determination to the parent drug.

These simplifying assumptions have a reasonable basis and will include the majority of cases.

Since the preparation of this review, a successful collaborative study has been conducted of the Manuel-Steller gas chromatographic method (11) and the gas chromatographic-mass spectrometric method of Suhre et al. (12) by Malanoski et al. (13) applicable to the determination of 0.1 ppm of sulfamethazine in hog muscle and liver. As a result of the urgency of the situation, the regulatory agencies assigned sufficient resources to solve the problem.

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PESTICIDE FORMULATIONS

Gas-Liquid Chromatographic Determination of Terbuthylazine Technical and Its Formulations: Collaborative Study

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Terbuthylazine technical and its 80% wettable powder were analyzed in a CIPAC collaborative study. The content and the identity were determined by gas-liquid chromatography on a column of 3% Carbowax K20M on Gas-Chrom Q, using di-n-pentylphthalate as the internal standard. Results obtained from 27 government, university, and industrial collaborators showed within-laboratory repeatability of 1.3% for the technical and 1.0% for the wettable powder. Reproducibility was 2.1% for both samples. The method has been adopted official first action.

A gas-liquid chromatographic (GLC) procedure for the determination of terbuthylazine (2-tert-butylamino-4-chloro-6-ethylamino-s-triazine) in technical material and formulations was collaboratively studied. The study was initiated by PAC-CH (Pesticides Analysis Advisory Committee Switzerland) to evaluate the precision and reliability of the method. An identity test was carried out simultaneously with the determination of content.

Collaborative Study

One sample each of terbuthylazine technical and 80% wettable powder (WP) formulation, together with standards, practice samples, and detailed guidelines, were sent to 31 collaborators in Europe and the United States. All collaborators were asked to perform the GLC analysis in duplicate using electronic peak area measurement. The participants were also requested to submit the raw data and the appropriate chromatograms.

This method was accepted as a full CIPAC method at the 23rd Annual Meeting of the Collaborative International Pesticide Analytical Council, 1979. The method was approved by the General Referee and Committee A and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* **64**, 422 (1981)

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Terbuthylazine (2-*tert*-Butylamino-4-chloro-6-ethylamino-*s*-triazine)

CIPAC-AOAC Method — Official First Action

6.B20 ***Principle***

Terbuthylazine is detd by gas chromatography using di-*n*-pentyl phthalate as internal std. Identity is confirmed simultaneously by comparing retention times with std.

6.B21 ***Standard Solutions***

(a) *Internal std soln.*—Weigh 4.0 ± 0.2 g di-*n*-pentyl phthalate, dil. to 1 L with CH_2Cl_2, and mix well. Std should be ≥98% pure and contain no impurities eluting at retention time of terbuthylazine.

(b) *Terbuthylazine calibration soln.*—Accurately weigh, in duplicate, ca 250 mg terbuthylazine ref. std of known purity (available from PAC-Switzerland, Swiss Federal Research Station, CH-8820 Waedenswil, Switzerland) into 100 mL g-s flasks. Pipet 50.0 mL internal std soln, (a), into each flask, stopper, and dissolve terbuthylazine by swirling.

6.B22 ***Preparation of Sample***

Accurately weigh sample contg ca 250 mg terbuthylazine into 100 mL g-s flask. Pipet in 50.0 mL internal std soln, (a), stopper, and shake 3 min. Let insol. materials settle and use supernate for injection.

6.B23 ***Gas Chromatography***

Use instrument equipped with flame ionization detector and 1.8 m × 2 mm id glass column packed with 3% Carbowax K 20M (Applied Science Laboratories, Inc.) on 80–100 mesh Gas-Chrom Q. Condition 24 h at 230° with N or He

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Table 1. Collaborative results for the analysis of terbuthylazine technical (%)

Coll.	Detn 1	Detn 2	$\bar{x}$
1	98.60	98.40	98.50
2	97.62	98.10	97.86
3	98.29	98.12	98.21
4	99.02	98.85	98.94
5	98.40	98.66	98.53
6	98.87	99.13	99.00
7	97.97	97.43	97.70
10	98.20	98.40	98.30
11	97.32	97.27	97.30
12	97.38	99.27	98.33
13	98.87	99.73	99.30
14	98.37	98.26	98.32
15	99.11	99.58	99.35
16	97.09	97.85	97.47
17	98.00	97.68	97.84
19	98.46	98.28	98.37
20	98.34	97.93	98.14
21[a]	(96.56)	(96.58)	(96.57)
22	97.08	97.94	97.51
23	97.63	97.60	97.62
24	98.23	98.01	98.12
25	97.48	96.68	97.08
26	97.15	97.26	97.21
27	98.06	97.94	98.00
28	98.80	97.40	98.10
29	97.16	97.59	97.38
30	98.97	99.49	99.23

[a] On the basis of the Nalimov test (significant at the 95% level) results were not included.

Table 2. Collaborative results for the analysis of 80% wettable powder formulations of terbuthylazine (%)

Coll.	Detn 1	Detn 2	$\bar{x}$
1	79.80	80.20	80.00
2	79.55	79.33	79.44
3	80.60	80.56	80.58
4[a]	(81.75)	(81.75)	(81.75)
5	80.76	81.24	81.00
6	79.50	78.56	79.03
7	79.75	80.09	79.92
10	79.80	79.30	79.55
11	79.18	79.45	79.32
12	78.86	79.14	79.00
13	79.90	80.12	80.01
14	79.19	80.49	79.84
15	80.39	81.26	80.83
16	79.31	79.55	79.43
17	79.37	79.39	79.38
19	80.35	80.34	80.35
20	79.83	79.98	79.91
21[a]	(78.38)	(78.29)	(78.34)
22	79.13	78.46	78.80
23	79.59	79.83	79.71
24[b]	(79.06)	(81.67)	(80.37)
25	81.10	81.08	81.09
26	79.98	79.23	79.61
27	80.39	80.37	80.38
28	79.00	79.70	79.35
29	79.31	79.16	79.24
30	81.14	80.82	80.98

[a] See footnote, Table 1.
[b] On the basis of the Bartlett test (significant at the 95% level) results were not included.

at ca 30 mL/min. Column must give baseline sepn between peaks of terbuthylazine and internal std; otherwise, prep. new column.

Operating conditions: temps (°)—inlet 250, column 210, detector 270; N or He carrier gas 35 mL/min; air and H as recommended for detector. For monitoring sepn, record chromatograms using suitable attenuation. Measure peak areas by electronic integration. Retention times (min)—terbuthylazine ca 6, di-*n*-pentyl phthalate ca 8.

6.B24 *Calibration*

Alternately inject 2 μL aliquots of the 2 calibration solns until calibration factors $F = W_c/R_c$ of 2 successive chromatograms vary ≤1%, where W_c = mg terbuthylazine std for calibration solns C_1 and C_2, resp.; R_c = peak area ratios of terbuthylazine/internal std. For the next steps, use only calibration soln C_1.

6.B25 *Determination*

Inject 2 μL calibration soln. Then make duplicate injections of sample followed by one injection of calibration soln. Individual calibration factors must lie within ±1%, otherwise repeat series of injections. Repeat for addnl samples.

6.B26 *Calculation*

Average calibration factors (F) preceding and following samples. Calc. and average peak area ratios of terbuthylazine/internal std of the 2 sample injections.

% Terbuthylazine = $R_s \times F_{av.} \times P/W_s$ where R_s = av. peak area ratio of the 2 sample injections; $F_{av.}$ = av. calibration factor; P = % purity of terbuthylazine std; and W_s = mg sample.

6.B27 *Identity Test*

Identity of terbuthylazine is confirmed if differences of retention times between terbuthylazine and internal std of sample and calibration solns do not deviate >0.2 min.

Results and Discussion

Results were received from 27 of 31 collaborators (Tables 1 and 2). All of the participants had performed the desired number of analyses. One collaborator had carried out the analysis twice. To reduce those data to standard form, one data pair was randomly chosen.

A wide variety of gas chromatographic instruments, integrators, and data systems were

Table 3. Averages, ranges, and standard deviation (SD) for collaborative results

Parameter	Technical	80% WP
No. of collaborators	26	24
No. of results	52	48
Range, overall	96.68–99.73	78.46–81.26
Average, overall	98.14	79.86
SD, absolute—repeatability	0.44	0.36
SD, absolute—overall[a]	0.72	0.72
SD, relative—repeatability	0.45	0.44
SD, relative—overall[a]	0.74	0.90
Repeatability, $r(95)$[b]	1.3	1.0
Reproducibility, $R(95)$[c]	2.1	2.1

[a] Reproducibility standard deviation: one assay, one laboratory. $s_x = \sqrt{s_L^2 + s_0^2}$.
[b] $r(95) = t \times \sqrt{2MS_0}$.
[c] $R(95) = t \times \sqrt{MS_0 + MS_L}$.

used. Four participants had no electronic integrator available and based their calculations on peak height measurements. These results were also used for statistical evaluation. The stationary phase was furnished by PAC. The columns prepared had between 790 and 3050 theoretical plates.

We reviewed the raw data. Based on the mean values, one result of the technical material and 2 results of the 80% WP formulation were rejected as outliers on the basis of the Nalimov test at the 95% level (1). After one collaborator's results for the 80% WP formulation are excluded, the data material is homogeneous according to the Bartlett test (2).

Table 3 contains averages and standard deviations as well as values for reproducibility $R(95)$, and repeatability, $r(95)$ (3). The analyses of variance (4) are given in Table 4.

The statistical treatment leads to the following conclusions: (1) The repeatability of the method is good. The corresponding absolute standard deviation is approximately 0.4%. Repeatability, $r(95)$, values were 1.3 for the technical and 1.0 for the wettable powder. (2) The reproducibility of the method is satisfactory. The corresponding absolute overall standard deviation is 0.7%. Reproducibility, $R(95)$, is 2.1 for the technical and the wettable powder. (3) Small differences exist in the mean values between laboratories, which are significant at the 95% level. However, the range of the mean values is satisfactory and in line with other collaborative studies.

The identity was generally confirmed by GLC.

The determination of the content did not lead to any major difficulties. Minor modifications of the GLC method such as slightly increasing or decreasing the column temperature to adjust retention times or resolution are common practice in GLC and may depend on column packing, apparatus, and so on.

Some remarks of the collaborators refer to the preparation of the internal standard stock solu-

Table 4. Analysis of variance of collaborative results

Source	Mean square	Degrees of freedom	F (found)	F (tabulated) (95%)
		Terbuthylazine Technical		
Between colls.	0.85	25		
Within colls.	0.19	26	4.36	1.94
Total	—	51		
		Terbuthylazine 80% WP		
Between colls.[a]	0.90	23		
Within colls.[b]	0.13	24	7.09	1.99
Total	—	47		

[a] $MS_L = 2 \times s_L^2 + s_0^2$.
[b] $MS_0 = s_0^2$.

tion and the calibration solution: The method requires pipetting 50.0 mL of a dichloromethane solution, which is in agreement with several official AOAC-CIPAC methods for triazines and other pesticides (5). Four co-workers questioned the accuracy of this procedure and recommended the use of a buret or a volumetric flask instead. One collaborator found the preparation of the stock solution not practical; he added the internal standard by direct weighing.

With regard to the GLC procedure, the requirement that the calibration factors should be within ±1% presented problems for one laboratory with deviations between 1 and 2%. Another co-worker could achieve the desired precision only by retaining the needle about 3 s in the injection port. Another participant found unusually large differences in the peak height ratios of the same calibration solution. It was also suggested that calibration standards be run in duplicate to ensure the most valid results, and that smaller injection volumes be used.

Conclusion

As the results reveal, the analytical method tested is rugged and accurate and yields reproducible results. A small yet statistically significant bias between laboratories has often been noted in collaborative studies. This may arise from inevitable differences in equipment, reagents, and skill of the operators. However, repeatability and reproducibility appear to be satisfactory. Only slight modifications to the method have been brought to our attention.

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Gas-Liquid Chromatographic Determination of Tetradifon Technical and Formulations: Collaborative Study

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The gas-liquid chromatographic determination of tetradifon technical and formulations was collaboratively studied in duplicate with 12 laboratories. Six samples were dissolved in dichloroethane with n-hexacosane as the internal standard, chromatographed on a column of 3% SE-52, and detected by flame ionization. The average coefficients of variation were 1.2% for the 2 technical samples, 1.6% for the 2 wettable powders, and 1.5% for the 2 emulsifiable concentrates. The method has been adopted official first action.

Tetradifon (4-chlorophenyl 2,4,5-trichlorophenylsulfon; Tedion V-18) is used to control spider mites on a wide variety of food, fiber, and ornamental crops. Infrared (1) and total chloride (2) methods have been reported; however, these are not specific enough. The gas-liquid chromatographic (GLC) procedure (2) is quick and precise, and easily separates interfering substances from the active ingredient.

Collaborative Study

Twelve laboratories were each sent 2 samples of 95% technical tetradifon, 2 samples of 20% wettable powders, and 2 samples of 8% emulsifiable concentrates along with a copy of the GLC method. Collaborators were instructed to analyze the samples in duplicate, each set on a different day and, if possible, by different analysts. For each collaborator, the sequence of sample analyses was randomly assigned in advance. Fresh standard solutions were to be prepared each day. Each determination was started with 2 standard solutions (at 2 concentration levels), and standards were repeated after every 3 samples.

Tetradifon Technical (4-Chlorophenyl 2,4,5-Trichlorophenyl Sulfone)

CIPAC-AOAC Method
Official First Action

6.B09 ***Principle***

Tetradifon is detd by flame ionization GLC, using *n*-hexacosane as internal std.

6.B10 ***Apparatus***

(**a**) *Gas-liquid chromatograph.*—Suitable for on-column injection; equipped with flame ionization detector.

(**b**) *Gas chromatographic column.*—3 mm id × 6 ft glass column packed with 3% SE-52 on 100–120 mesh Chromosorb W-HP. Operating conditions: injector and detector 250°, column 230°, N or He carrier gas flow ca 35 mL/min. Approx. retention times for tetradifon and *n*-hexacosane = 8.6 and 10 min, resp.

6.B11 ***Reagents***

(**a**) *Internal std soln.*—Accurately weigh 0.30 g pure *n*-hexacosane into 100 mL vol. flask. Dil. to vol. with 1,2-dichloroethane and mix.

(**b**) *Tetradifon std soln.*—Accurately weigh 0.100 g pure tetradifon (available from Philips-Duphar B.V., Weesp, The Netherlands; or Chemical and Biological Investigations, Environmental Protection Agency, Beltsville, MD 20705) into 100 mL vol. flask, pipet 20.0 mL internal std soln, (**a**), into flask, dil. to vol. with dichloroethane, and mix.

6.B12 ***Preparation of Sample***

(**a**) *Technical tetradifon.*—Accurately weigh 100 mg sample into 100 mL vol. flask. Pipet 20.0 mL internal std soln, (**a**), into flask, dil. to vol. with dichloroethane, and mix thoroly.

(**b**) *Wettable powder.*—Accurately weigh sample contg 100 mg tetradifon into 250 mL g-s flask.

The method presented was accepted as a full CIPAC method (document 2600) at the 22nd meeting of the Collaborative International Pesticides Analytical Council, 1978. The method was approved by the AOAC General Referee and Committee A and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* **64**, 421 (1981)

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Pipet in 20.0 mL internal std soln, (a), and 80.0 mL dichloroethane. Heat on H_2O bath 5 min, cool, and mix thoroly. Transfer ca 40 mL soln to centrif. tube, and centrif. 10 min.

(c) *Emulsifiable concentrates.*—Accurately weigh sample contg 100 mg tetradifon into 100 mL vol. flask. Pipet 20.0 mL internal std soln, (a), into flask. Dil. to vol. with dichloroethane and mix thoroly.

6.B13 ***Determination***

Adjust chromatographic conditions to give ca ½ FSD for 2 μg tetradifon. Inject 2 μL portions of std soln, (b), until response factor (F) varies <1% for successive injections. Inject 2 μL sample soln, and measure peak hts and retention times for both std and sample.

6.B14 ***Calculation***

$$F = (I' \times T \times P \times 5)/(I \times S \times 100)$$

where I' and I = peak ht × retention time of internal std and tetradifon, resp.; T = g tetradifon in calibration soln; P = % purity of std; S = g internal std. (Response factor is ca 1.5.)

$$\% \text{ Tetradifon} = (I \times S \times F \times 100)/(I' \times W \times 5)$$

where W = g sample.

Results and Recommendations

Although the use of response factors was prescribed in the instructions, tetradifon content was calculated in various ways; therefore, results were recalculated in the following way: The first 3 samples were calculated using the mean of the 2 response factors of the first standard run, and the second series of 3 samples were calculated using the mean of the second standard run. The third standard run served as a check for stability. Collaborator 8 reported 2 response factors per standard solution. His results were recalculated, using the first value.

Recalculated individual results are presented in Table 1. Collaborator 7 did not report duplicate analyses. Although his results were in agreement with the other data, they were excluded in the statistical analysis. The ranking test as described by Youden (3) was applied to the remaining data. This revealed an extreme score for the results of Collaborator 11 ($P = 0.05$). This collaborator also found large differences between response factors. For these reasons, his results were excluded from the final statistical analysis.

The data from the remaining 10 laboratories were examined for homogeneity of experimental variation. The samples consisted of 3 matched pairs, so we applied the tests for constant within-sample (between-laboratory) and constant within-laboratory variation to the 3 pairs of samples separately (3).

For the tetradifon technical pair, the test for homogeneity within-sample and within-laboratory variation was not significant at the 5% level; therefore, estimates of S_r and S_b were obtained on the basis of combined data. For the wettable powder pair, the test for homogeneity of the within-sample variation also was not significant at the 5% level. The test for homogeneity of the within-laboratory variation was just significant at the 5% level. This was caused by the relevant extreme value of Sample 2-4 in Laboratory 12. We decided that this difference was not serious enough to justify exclusion of these results. For the emulsifiable concentrate pair, the between-laboratory variations of the 2 samples were not homogeneous; thus the estimates for S_r, S_b, and S_d were calculated separately for Samples 3-2 and 3-4. Laboratory 12 reported a large difference between replicates on Sample 3-2 (P two-sided is 0.05). Because 8.14% was very low, and the repeatability was poor in comparison with other results, we decided to exclude the results of Sample 3-2 from Collaborator 12. The estimates of S_r for the 2 samples suggest that the within-laboratory errors may be considered equal; therefore, these estimates were pooled, and the combined estimate was reported as CV = 1.5%.

Reproducibility for the emulsifiable concentrate was very poor compared with that for the other 2 products. Considering the results for the other products and the good repeatability, it is not expected that this was caused by the method. From experiments with blank formulations and comparisons of retention times of major impurities of the technical material, we know that no compounds were present with retention times close to those of the active ingredient and the internal standard. This means that a difference in the resolution of the column was not the cause. Further study was carried out in 3 laboratories after the collaborative test was finished, with spare samples stored for approximately one year in the same bottles used for the collaborative test. These results were also higher compared with the analyses at the start of the test (3% w/w for Sample 3-2 and 1% w/w for Sample 3-4); however, in the original large bottle of Sample 3-2, we found the same content of active ingredient as before the test. On the basis of the results of this additional study, we believe that the differ-

Table 1. Collaborative results for determination of tetradifon (%)

	Technical		Wettable powder		Emulsifiable conc.	
Coll.	1-1	1-5	2-1	2-4	3-2	3-4
1	97.96	94.43	19.01	18.98	8.75	8.78
	98.84	95.08	19.13	18.96	8.93	8.93
2	98.87	95.46	19.07	19.13	9.07	9.13
	99.01	94.49	19.06	19.17	9.09	9.03
3	96.64	93.04	18.59	19.00	8.96	9.01
	97.64	93.15	19.00	19.07	8.85	9.01
4	97.94	96.29	19.19	19.54	9.65	9.30
	97.21	93.82	19.23	18.79	9.29	9.74
5	98.20	94.00	19.00	19.83	9.64	9.26
	97.72	93.60	18.40	19.10	9.52	9.30
6	96.69	93.47	18.76	19.17	11.00	9.47
	97.18	92.17	18.97	19.06	10.99	9.67
7[a]	98.26	92.67	18.36	18.57	10.13	9.88
8	96.46	93.82	18.73	19.03	11.40	9.76
	98.37	95.11	18.99	18.98	11.26	9.68
9	98.65	94.99	19.04	18.91	10.21	9.87
	99.54	94.97	19.17	19.48	9.79	9.58
10	97.09	96.00	19.01	19.17	11.07	9.94
	97.95	94.05	19.67	19.20	10.93	9.97
11[b]	95.89	93.88	18.16	18.90	9.13	8.29
	92.92	94.43	17.75	18.69	9.68	9.40
12	100.41	95.24	19.10	18.67	8.89	8.91
	101.75	95.05	18.88	19.89	8.13[c]	8.98
Av.	98.21	94.41	19.00	19.16	9.91	9.37
S	1.20		0.30		0.98	0.39
CV, %	1.2		1.6		9.9	4.2
DF	21		29		8	10
S_r	0.86		0.30		0.15	0.13
CV, %	0.89		1.6		1.5	1.4
DF	29		29		9	10
S_b	0.84		0		0.96	0.37
CV, %	0.87		0		9.7	4.0
DF	5		—		7	7
S_r (combined)					0.14	
CV, %					1.5	
DF					19	

[a] Rejected because of only one determination; not included in statistical analysis.
[b] Excluded from statistical analysis because of extreme ranking test score.
[c] Rejected because of poor reproducibility.

ence between the collaborators is certainly caused by diffusion of the organic solvent through the plastic cap of the bottle. Because Sample 3-2 had a larger and thinner cap than Sample 3-4, it explains why Sample 3-2 showed a more pronounced evaporation than Sample 3-4; thus, depending on date of the analyses and storage conditions, the samples would be more or less evaporated.

The results show that this method is suitable for determining tetradifon in technical products, wettable powders, and possibly emulsifiable concentrates. The results for the emulsifiable concentrates seemed to be less satisfactory; however, in our opinion, it was not caused by method failure but probably by diffusion of solvents. We are convinced that this method can also be applied for emulsifiable concentrates, considering the good repeatability for the emulsifiable concentrates and the good results for the other 2 products.

It is recommended that the GLC method be adopted official first action for the determination of tetradifon in technical products, wettable powders, and emulsifiable concentrates.

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PESTICIDE RESIDUES

Gas-Liquid Chromatographic Determination of Aniline Metabolites of Substituted Urea and Carbamate Herbicides in Aqueous Solution

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A simple gas-liquid chromatographic (GLC) method has been developed which provides sensitivity and specificity for the analysis of complex mixtures of the commonly occurring herbicide metabolites aniline, 3-chloroaniline, 4-chloroaniline, 4-bromoaniline, and 3-chloro-4-methylaniline. All of these anilines react with acetic anhydride directly in basified aqueous solution. Further reaction of the acetylated anilines with trifluoroacetic anhydride gave diacyl derivatives which were readily resolved by gas chromatography. The structures of the *N*-acetylated and *N*-trifluoroacetylated derivatives of benzylamine (internal standard) and the anilines were confirmed by GLC-mass spectrometry. In distilled water the minimum detectable concentrations of aniline and the substituted anilines, using electron capture GLC, are 0.1 nmole/100 mL and 0.05 nmole/100 mL, respectively. The detection limit for the anilines is 1 nmole/100 mL distilled water, using GLC with flame ionization detection. The technique was applied to the determination of anilines added to urine samples obtained from the general population.

Aniline and a number of substituted anilines are commonly used in the paint, dye, and drug industries (1). They are introduced into the environment directly as industrial waste, and indirectly by the combustion of plastics and urethane products or as the reduced form of nitrobenzene compounds (2). The degradation of *N*-phenylcarbamate and *N*-phenylurea herbicides (Table 1) in soils (3–6), environmental water systems, and humans (7) is the most common source of anilines. Although aniline biodegradation products have no herbicidal properties (6), they are more toxic than the parent compounds and persist in the environment strongly bound to soil organic matter (6, 7). In addition, they may undergo further conversion to persistent and carcinogenic azo metabolites (8, 9). In view of the toxicity of these anilines, the development of sensitive analytical techniques for their detection in water and biological fluids is necessary.

Anilines have been determined by gas-liquid chromatography (GLC) (3, 10–13) and high performance liquid chromatography (HPLC) (2, 14). Using solvent programming, Lores et al. (2) separated and analyzed a mixture of halogenated anilines by HPLC without derivatization. This technique, however, lacked sensitivity when UV detection was used. Bradway and Shafik (12) assessed the merits of a number of reagents used in the preparation of derivatives for electron capture GLC. Even though a complex mixture of herbicide metabolites could be resolved following derivatization with 4-chloro-α,α,α-trifluoro-3,5-dinitrotoluene, the reagent failed to give clean chromatograms and was unsuitable for trace analysis. A chloroacetic anhydride derivative of 3,4-dichloroaniline has been prepared (11) for quantitative analysis, but in this procedure, derivatization followed extraction of the aniline into chloroform. The direct acetylation of anilines in water has not been reported.

Acetic anhydride has been used successfully for the preparation of acetate derivatives of a number of phenols (15), nitrophenols (16), and aminophenols (17) in aqueous solution. The direct aqueous acetylation of the aniline degradation products listed in Table 1 proceeds rapidly to completion at room temperature to produce acetanilides which could be quantitatively extracted from water by using a suitable solvent. Further reaction of the amides with trifluoroacetic anhydride produced diacyl derivatives (*N*-acetyl, *N*-trifluoroacetyl), which are sensitive to GLC analysis using either a flame ionization detector (FID) or an electron capture detector (ECD). Aniline, benzylamine (internal standard), 3-chloroaniline, 4-chloroaniline, 3-chloro-4-methylaniline, and 4-bromoaniline can be completely separated on a conventional packed column. Using capillary chromatography and an ECD, the minimum detectable concentration of aniline and each of the substituted

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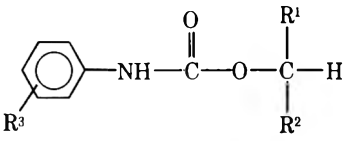
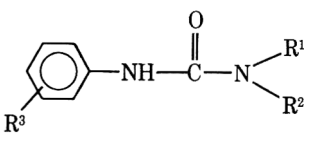
Table 1. Decomposition of certain herbicides to aniline degradation products

I



N-Phenylcarbamate

II



N-Phenylurea

Class	Common name	R^1	R^2	R^3	Degradation product
I	Propham (IPC)	$-CH_3$	$-CH_3$	$-H$	aniline
I	Carbetamide	$-CH_3$	$-CNHCH_2CH_3$ (O)	$-H$	
II	Fenuron	$-CH_3$	$-CH_3$	$-H$	
II	Siduron	$-H$		$-H$	
I	Chloropropham	$-CH_3$	$-CH_3$	3-Cl	3-chloroaniline
I	Barban	$-C{\equiv}CCH_2Cl$	$-H$	3-Cl	
II	Monolinuron	$-CH_3$	$-OCH_3$	4-Cl	
II	Buturon	$-CH_3$	$-CH(CH_3)C{\equiv}CH$	4-Cl	4-chloroaniline
II	Monuron	$-CH_3$	$-CH_3$	4-Cl	
II	Metabromuron	$-CH_3$	$-OCH_3$	4-Br	4-bromoaniline
II	Chlorotoluron	$-CH_3$	$-CH_3$	3-Cl, 4-CH_3	3-chloro-4-methylaniline

anilines is 0.1 nmole/100 mL and 0.05 nmole/100 mL distilled water, respectively. The technique can be applied easily to both large and small volumes of aqueous solution. It has been suggested (14) that the determination of aniline and substituted aniline levels in urine can be used as an index of exposure to carbamate and urea pesticides. The method described here is applicable to the analysis of urine samples containing trace amounts of aniline and substituted anilines. No currently available GLC or HPLC method can simultaneously provide sensitivity with complete component resolution.

METHOD

Reagents and Samples

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

(b) *Solvents.*—Methylene chloride, glass-distilled before use.

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

(d) *Aniline standard solutions.*—Store individually prepared solutions (0.01mM in 95% ethanol) of aniline, 3-chloroaniline, 4-chloroaniline, 3-chloro-4-methylaniline, 4-bromoaniline, and benzylamine (internal standard) in glass-stoppered bottles at 4°C.

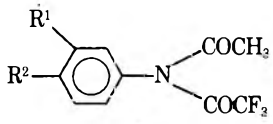
(e) *Urine samples.*—First void samples are obtained from persons in the general population without known occupational exposure to industrial chemicals.

Apparatus

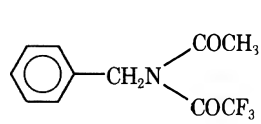
(a) *ECD gas chromatograph.*—Hewlett-Packard Model 5830A equipped with 15 mCi ^{63}Ni ECD, Model 18850A integrator, and 10 m × 0.25 mm id SP-2100 WCOT glass capillary column (J & W Scientific, Orangevale, CA). Operating conditions: temperatures (°C)—injector 250, column programmed from 80 to 120 at 10°/min, detector 250; helium carrier gas at 7 psi; 10% methane in argon make-up gas 36 mL/min.

(b) *FID gas chromatograph.*—Hewlett-Packard Model 5702A with Model 3380A integrator. Column 1: 1.68 m × 4 mm id glass column packed with 3% OV-17 on 80–100 mesh Chromosorb W. Column 2: 0.84 m × 4 mm id glass column packed with 0.3% Carbowax on glass beads. Operating temperatures (°C)—Column 1, for diacyl derivative analysis 140, for analysis of diacyl derivative decomposition column programmed from 170 to 200 at 8°C/min; Column

Table 2. Diagnostic ions in spectra of *N*-acetyl, *N*-trifluoroacetyl derivatives of substituted anilines (I) and internal standard, benzylamine (II)



I



II

Class	Derivatives R1	Derivatives R2	m/z (% relative abundance)
I	—H	—H	231 (absent) [M^{+}], 189(100), 120(42), 92(10), 69(10), 43(84)
I	—Cl	—H	267(1.5), 265(4)[M^{+}], 225(35), 223(100), 156(9), 154(30), 128(5), 126(2), 43(71)
I	—H	—Cl	265(3)[M^{+}], 225(33), 223(100), 156(8), 154(30), 128(9), 126(12), 43(76)
I	—Cl	—CH_3	281(2), 279(6)[M^{+}], 239(34), 237(100), 170(8), 168(28), 142(8), 140(12), 69(8), 43(60)
I	—H	—Br	311(5), 309(6)[M^{+}], 269(96), 267(100), 200(36), 198(34), 172(28), 170(20), 69(20), 43(95)
II			245(5)[M^{+}], 203(100), 202(75), 106(37), 91(28), 69(15), 43(91)

2, 190; detector and injector 250 for both columns. Helium carrier gas 60 mL/min.

(c) *Gas chromatograph-mass spectrometer.*—Mass spectra are obtained using Columns 1 and 2 (parameters described in (b) above) on a combined Hewlett-Packard Model 5710A gas chromatograph-Model 5981A mass spectrometer coupled to a Model 5934A data system. Mass spectrometry (MS) scan conditions: scan speed, 100 amu/s; band width, 430 Hz; electron energy, 70 eV; ion source temperature 180°C. Separator temperature is the same as the oven temperature.

Preparation of Spiked Water Samples

Two calibration graphs are constructed for each aniline by using the internal standard method: one graph for the packed 3% OV-17 column with flame ionization detection and the second for the SP-2100 capillary column with electron capture detection. The minimal detectable concentration of each aniline for the above GLC systems is also determined.

(a) *FID calibration and detection limit.*—Spike 100 mL distilled water samples with 50 nmoles benzylamine and 1–100 nmoles of each aniline listed in (d), *Reagents.*

(b) *ECD calibration and detection limit.*—Spike 100 mL distilled water samples with 5 nmoles benzylamine and 0.05–20 nmoles of each aniline.

Preparation of Spiked Urine Samples

Spike 5 mL urine sample in 15 mL Teflon®-lined screw-cap test tube with anilines as described above. Add 0.2 mL concentrated H_2SO_4, seal tube tightly, and hydrolyze 1 h in boiling water bath. Cool hydrolyzed sample, add internal standard benzylamine, and extract twice, each time by shaking 2 min with 2 mL methylene chloride. Discard methylene chloride extracts, neutralize urine sample with 1.2 mL 10N NaOH, and proceed with derivatization.

Derivatization

(a) *Aqueous acetylation and extraction.*—Acetylate the anilines in 100 mL spiked distilled water sample by adding 500 μL acetic anhydride and 5 g $NaHCO_3$ as previously described (15). Similarly, add the anilines to 5 mL urine sample and react with 250 μL acetic anhydride and 0.5 g $NaHCO_3$. After evolution of carbon dioxide ceases, extract acetates by shaking water and urine samples 2 min with 10 mL and 2 mL, respectively, of methylene chloride. Remove trace amounts of moisture in methylene chloride by passing extract 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 extract to 100 μL by using gentle stream of nitrogen.

(b) *Trifluoroacetylation.*—Add 50 μL trifluoroacetic anhydride (TFAA) to 100 μL methylene chloride extract prepared above, shake vigorously 15 s, and let react at room temperature 15 min. (The usual method of neutralizing excess TFAA with alkaline buffer causes decomposition of the diacyl derivatives. Furthermore, the derivatives are volatile and subject to uncontrolled loss if the solvent is evaporated to dryness.) To avoid evaporative losses and to remove traces of TFAA, add 1 mL cyclohexane to reaction mixture and evaporate sample to 300 μL by using stream

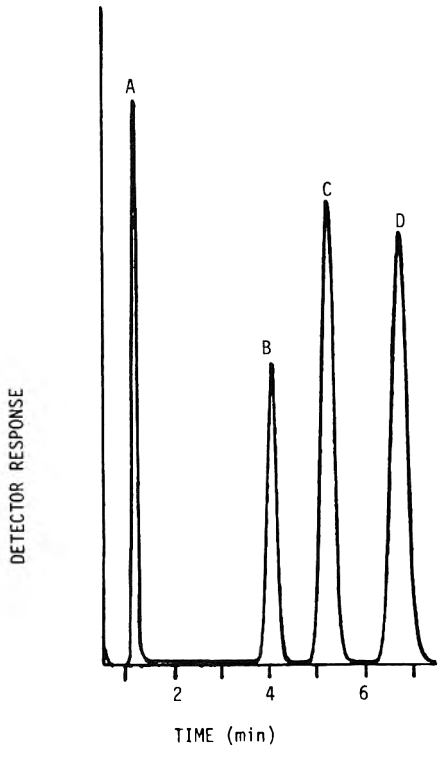


Figure 1. Computer-reconstructed total ion trace of *N*-acetylated anilines, from 0.2% Carbowax column. Peak identification of acetylated derivatives: A, benzylamine; B, 4-chloroaniline; C, 3-chloro-4-methylaniline; D, 4-bromoaniline.

of nitrogen. Inject 1 μL sample onto capillary column for ECD quantitation. For GLC with FID, add 0.1 mL cyclohexane to reaction mixture, reduce volume to 20 μL, and inject 1 μL onto packed column.

Mass Spectral Data

The mass spectral data shown in Table 2 are consistent with the assigned structures of the diacyl derivatives (i.e., *N*-acetylated and *N*-trifluoroacetylated) of aniline, 3-chloroaniline, 4-chloroaniline, 4-bromoaniline, 3-chloro-4-methylaniline, and benzylamine.

Results and Discussion

N-Phenylcarbamate and *N*-phenylurea herbicides have the desirable characteristics of low mammalian toxicity and rapid environmental degradation. They are readily biodegraded to aniline and the substituted anilines listed in Table 1. The presence of these anilino compounds in the environment is important (7), particularly in view of their resistance to biodegradation. Suess et al. (18) studied the metabolism of several anilines by 4 different soil types. When examined over a 10-week period and using ppm concentration levels, aniline, 4-chloroaniline and 3,4-dichloroaniline were degraded 16.2–26.3, 12.3–17.2, and 3.9–11.7%, respectively. A strong soil adsorption effect in combination with slow degradation results in the accumulation of chloroanilines in the soil and may extend their residual life up to 10 years (4).

Anilines are important constituents of industrial effluents recycled for agricultural use.

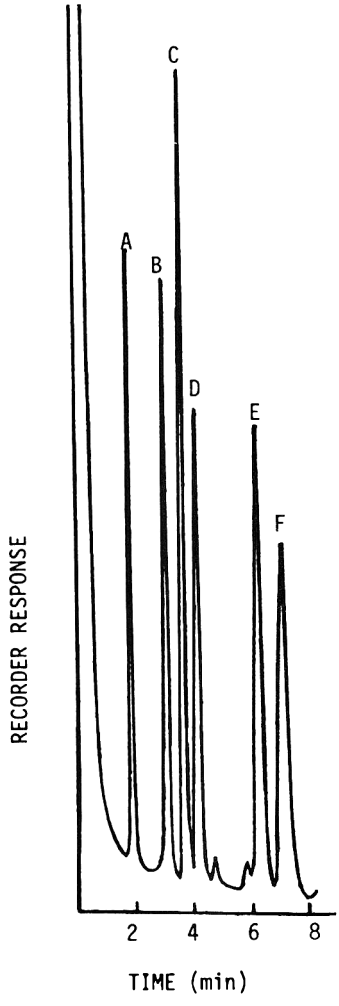


Figure 2. Gas chromatographic separation of *N*-acetyl-*N*-trifluoroacetylanilines on 3% OV-17 column. Peak identification of derivatives: A, aniline; B, benzylamine; C, 3-chloroaniline; D, 4-chloroaniline; E, 3-chloro-4-methylaniline; F, 4-bromoaniline. Fifty nmoles of each compound in 100 mL water was acetylated and trifluoroacetylated as described in text.

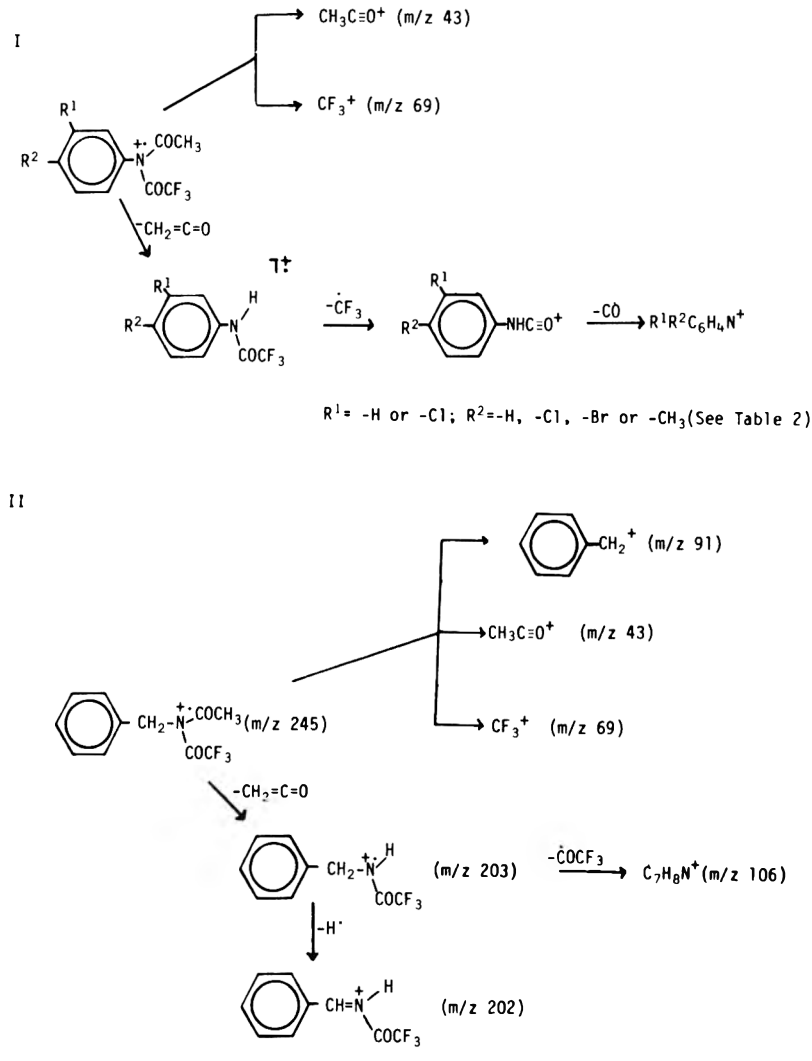


Figure 3. Mass spectral fragmentation patterns of (I) derivatized anilines and (II) derivatized benzylamine.

Trace quantities of aniline are found in plants a few hours after irrigation with water from river systems which receive this type of discharge. Low levels of aniline and other amines initially affect the turgor of the plants, induce chlorosis, and finally depress growth (1). Both the Soviet Union and the U.S. Environmental Protection Agency have introduced guidelines for the maximum permissible concentration of organics in drinking water. In the Soviet Union, the specific toxicological limits for aniline and 3- and 4-chloroaniline have been set at approximately 100 nmoles/100 mL and 155 nmoles/100 mL, respectively (19). Although the United States has not yet established specific concentration levels for these compounds, aniline and 2-chloroaniline are included in the consensus voluntary reference compounds list (20). The list includes known water pollutants for which present methodology is considered inadequate.

In this study, direct acetylation of an alkaline ($NaHCO_3$) aqueous solution by acetic anhydride completely converted the trace amounts of added aniline, benzylamine, 3-chloroaniline, 4-chloroaniline, 3-chloro-4-methylaniline, and 4-bromoaniline to their respective acetates. The *N*-acetates were efficiently extracted from aqueous solution into methylene chloride and could be stored for as long as one week at 4°C with virtually no decomposition. Following this simple

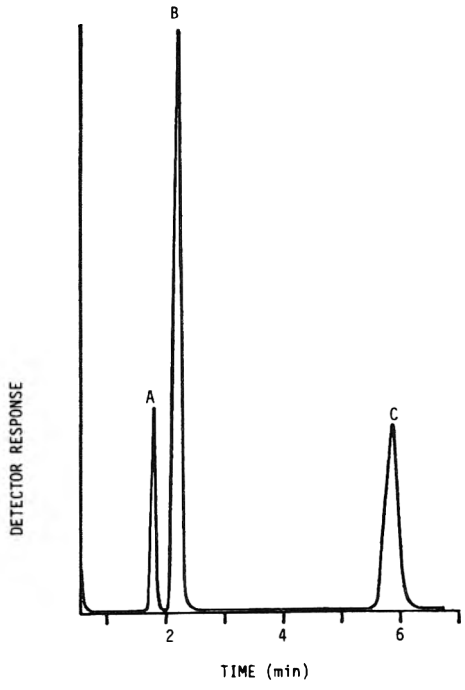


Figure 4. Computer-reconstructed total ion trace of decomposition products of *N*-acetyl-*N*-trifluoroacetyl-3-chloroaniline, following 14 h storage at 4°C. Peak identification: A, *N*-trifluoroacetyl-3-chloroaniline; B, *N*-acetyl-*N*-trifluoroacetyl-3-chloroaniline; C, *N*-acetyl-3-chloroaniline.

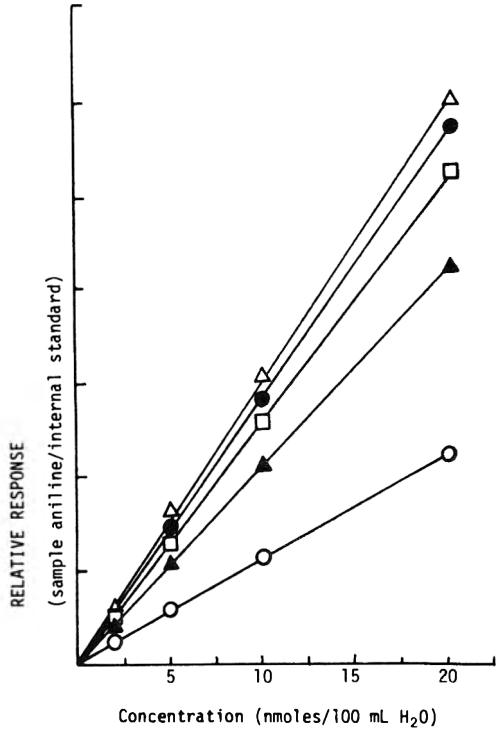


Figure 5. ECD calibration graphs for diacylated aniline derivatives, 1-20 nmoles in 100 mL distilled water. ○, aniline; ▲, 3-chloroaniline; ●, 4-chloroaniline; □, 3-chloro-4-methylaniline; △, 4-bromoaniline.

one-step acetylation/extraction procedure, 3-chloro-4-methylaniline could easily be separated from 4-bromoaniline (Figure 1) on a 0.2% Carbowax column. *N*-Acetyl-3-chloroaniline and *N*-acetyl-4-chloroaniline still had virtually identical retention times and could not be resolved.

The acetylated aniline derivatives were further reacted with trifluoroacetic anhydride. As shown in Figure 2, resolution of the *N*-acetyl-*N*-trifluoroacetyl anilines and benzylamine was complete within 7 min on a 3% OV-17 column run under isothermal conditions at 140°C. The trifluoroacetylation reaction proceeds to greater than 97% completion for all 5 anilines. The residual acetanilides, which are present following trifluoroacetylation, are much less volatile and do not interfere with the GLC analysis of the *N*-acetyl-*N*-trifluoroacetyl derivatives. The identities of the compounds giving rise to each peak in Figure 2 were confirmed by combined GLC-MS, and plausible fragmentation pathways are shown in Figure 3. The diacylated anilines are stable for 2-3 h; however, only 20-50% of the diacyl compounds remain after storage at 4°C for 14 h. *N*-Acetanilides and *N*-trifluoroacetanilides are present as decomposition products (Figure 4).

Calibration graphs for each of the derivatized anilines in distilled water were linear over the 5-100 nmoles/100 mL concentration range for the OV-17 column and flame ionization detection. The minimum detectable concentration was 1 nmole/100 mL. Because only 1 μL of the final derivative preparation (20 μL) was chromatographed, this represents an injection of 0.05 nmole "on column." The use of an ECD in combination with a capillary column greatly enhances the sensitivity of the procedure. The calibration graphs obtained (Figure 5) were linear over the concentration range 1-20 nmoles for each of the 5 anilines dissolved in 100 mL distilled water. The minimum detectable concentrations for aniline and each of the substituted anilines were 0.1 nmole/100 mL and 0.05 nmole/100 mL, respectively. This is a substantial improvement over the ECD-GLC limit be-

tween 3.2 and 6.3 nmoles/100 mL natural water reported (11) for 3,4-dichloroaniline following derivatization with chloroacetic anhydride. Our ECD detection limit represents an "on column" injection of 0.33 pmole aniline and 0.17 pmole substituted anilines.

Quantitation of trace amounts of pollutants in urine is hampered by the presence of endogenous urine components. However, application of the described analytical procedure to a 5 mL aliquot of hydrolyzed urine previously spiked with 100 nmoles benzylamine and 50 nmoles of each aniline derivative gave satisfactory results (Figure 6-I) from GLC with FID. The derivatized extract of a normal hydrolyzed urine sample (Figure 6-II-blank) did not contain detectable herbicide metabolites or large peaks which would interfere with the quantitation of 3- and 4-chloroaniline, 4-bromoaniline, or 3-chloro-4-methylaniline. At very low concentrations requiring high GLC detector gain, the aniline peak may be located in the solvent tail of some samples.

Present GLC and HPLC methodologies for the quantitation of aniline and haloaniline mixtures in dilute aqueous solutions are inadequate for several reasons. Many electron capture-sensitive derivatives prepared to improve GLC detection sensitivity do not allow simultaneous resolution of the aniline herbicide metabolites shown in Table 1. A recent publication (12) stated that of the many derivatives prepared using spiked distilled water, only those obtained by reaction with pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride gave clean chromatograms at low concentrations. PFPA was considered the reagent of choice for analysis even though derivatives of 3-chloroaniline and 4-bromoaniline could not be resolved from 4-chloroaniline and 3-chloro-4-methylaniline, respectively. Anilines have been analyzed by HPLC without prior extraction and derivatization. Lores et al. (2) were able to separate 3- and 4-chloroaniline by using solvent programming but their study did not include 3-chloro-4-methylaniline. In addition, quantities greater than 10 ng per injection were required for detection by UV. Using ECD, the detection limits and on-column sensitivities of our GLC technique compare favorably with those obtained by HPLC (2) with electrochemical detection. The HPLC on-column detection limits for aniline, 3-chloroaniline, 4-chloroaniline, and 4-bromoaniline were 2.1–2.6 pmoles, but without solvent programming, resolution of a complex aniline mixture was no longer possible. Analysis of the haloanilines in Table 1 required 2 HPLC runs using different solvent systems, and aniline was obscured in the solvent front of both mobile phases. If the components of a complex mixture are to be separated and identified unequivocally using HPLC, the sensitivity afforded by the use of an electrochemical detector must be sacrificed. In contrast, our derivatization procedure for GLC not only provides improved sensitivity for ani-

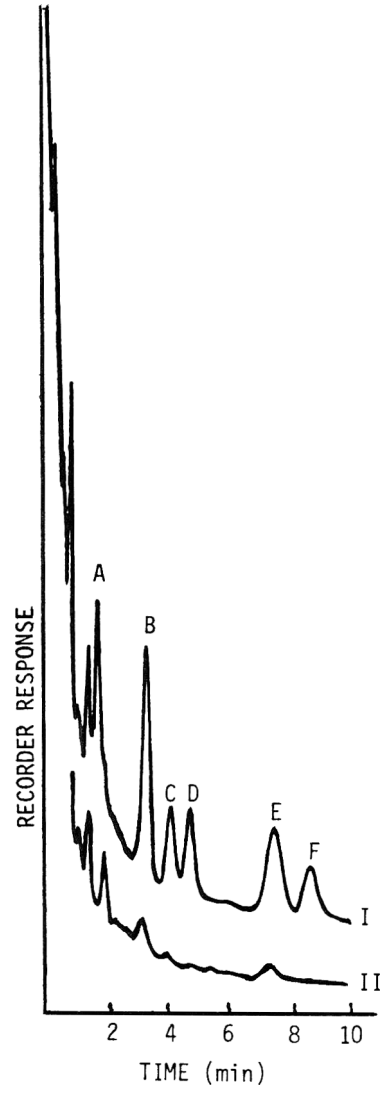


Figure 6. Gas chromatographic analysis of derivatized extracts of hydrolyzed urine samples (5 mL) using a 3% OV-17 column with FID detection. (I) Urine sample spiked with 50 nmoles of each aniline and 100 nmoles of benzylamine. (II) Blank urine sample. Identity of each peak is given in Figure 2.

lines but also allows the analysis of complex aniline mixtures which were unresolved when other analytical procedures were utilized.

Acknowledgment

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Portable and Sensitive Detector Strips for Rapid Detection of Organophosphorus, Mercury, Copper, Cadmium, and Silver Compounds

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Portable and sensitive detector strips developed for rapid detection of organophosphorus, mercury, copper, cadmium, and silver compounds are inexpensive, indefinitely stable, and easy to use. The strips require mild moistening with distilled water and warming for the reaction involving cholinesterase and succinate dehydrogenase inhibition by organophosphorus and heavy metal compounds, respectively. The detector strips, designated A, B, and C, can be combined to detect heavy metals and organophosphates as their oxygen analogs at nanogram concentrations in 5-10 min with little cleanup, so the strips can be used under field conditions.

Cholinesterase inhibition methods are used to detect organophosphorus compounds at nanogram concentrations by thin layer chromatography (1-4). These enzymatic methods are sensitive and specific (5-7) compared with chemical methods of estimation. Recently, the application of succinate dehydrogenase enzyme inhibition was reported (8) for detection and determination of heavy metal compounds of mercury, copper, and cadmium by paper and thin layer chromatography. The method is simple, rapid, sensitive, and useful for separation, identification, and determination. The portable detector strips reported in the present paper may be used under field or laboratory conditions for routine detection of organophosphorus compounds and/or heavy metals. The portable strips are inexpensive, can be stored indefinitely, and are easy to use.

METHOD

Apparatus and Reagents

Use analytical grade reagents.

(a) *Parathion (O,O-diethyl O-p-nitrophenyl phosphorothioate).*—99% pure (Ciba Geigy Ltd, Basel, Switzerland). Prepare required concentration in acetone. Prepare paraoxon through oxidation by exposing parathion to bromine vapors on Whatman No. 3 paper strip and extract repeatedly in acetone as described earlier (5, 7).

(b) *Dimethoate (O,O-dimethyl-S-[(methylcarbamoyl)methyl] phosphorodithioate).*—95% pure (Rallis India Ltd, Bombay, India). Prepare required concentration in acetone. Prepare omethoate through oxidation by exposing dimethoate to bromine vapors as described above.

(c) *Fenitrothion (O,O-dimethyl O-(4-nitro-m-tolyl) phosphorothioate).*—95% pure (Rallis India Ltd). Prepare fenitrooxon as described above.

(d) *Metals.*—Prepare required concentration of $HgCl_2$, $CuSO_4$, $CdSO_4$, and $AgNO_3$ in distilled water.

(e) *1-Naphthyl acetate substrate solution.*—Prepare 1% 1-naphthyl acetate (Sigma Chemical Co., St. Louis, MO 63178) in acetone.

(f) *Fast Blue B.*—Prepare 1% in distilled water.

(g) *Sodium succinate substrate-2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) mixture.*—Prepare 0.8% INT (BDH, India) in distilled water. Prepare 2.5% sodium succinate (BDH, Poole, UK) in distilled water. Mix reagents (1 + 1).

(h) *N-Methyl phenazonium methosulfate (PMS).*—Prepare 0.1% PMS in distilled water.

(i) *Enzyme suspension.*—Prepare 2% horse liver acetone powder suspension (Sigma Chemical Co.) in acetone by homogenizing with mortar and pestle.

(j) *Detector strip A.*—Cut 7 × 2.5 cm Whatman No. 3 filter paper. Dip 10 strips in horse liver acetone powder suspension and let dry in horizontal position at room temperature without contact with substrate. About 10 mL enzyme suspension may be used for 10 strips.

(k) *Detector strip B.*—Dip 7 × 2.5 cm Whatman No. 3 filter paper strip in 1% 1-naphthyl acetate solution. Remove and air dry. Again dip in 1% Fast Blue B solution. Let dry at room temperature or in 50°C hot air oven in horizontal position.

(l) *Detector strip C.*—Dip 7 × 2.5 cm Whatman No. 3 filter paper strip in sodium succinate substrate-INT mixture. Remove and air dry at room temperature or in 50°C hot air oven.

Preserve A, B, C detector strips separately in polythene covers and wrap with black paper.

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Store strips in moisture-free glass containers (do not use desiccator) at room temperature. A and C strips are stable indefinitely. B strip is stable 15 days.

Detection of Organophosphorus Compounds

Apply 0.5 μg methyl paraoxon, omethoate, or fenitrooxon with micro capillary pipet on 2.5 sq. cm portion of detector strip A; dry spot by gentle air stream. Place this strip horizontally on clean micro glass slide and spray distilled water as fine mist until it is wet. Do not over-spray (to prevent leaching). Warm slide and strip 2 min at 40°C, or lightly warm with cigarette lighter flame (suitable for field conditions). Place 2.5 sq. cm portion of detector strip B on another clean micro glass slide and moisten as described above; immediately sandwich A and B strips between 2 micro glass slides, placing A strip below B strip. Organophosphorus compounds appear as white spot against magenta background. As light tinge of color appears, separate strips to avoid masking white inhibition spot.

Detection of Mercury, Copper, Cadmium, and Silver Compounds

Apply 1 μg $HgCl_2$, $CuSO_4$, $CdSO_4$, or $AgNO_3$ with micro capillary pipet on 2.5 sq. cm portion of A strip and moisten strip as described above. Warm strip 3 min at 60°C. Strip should not be completely dry.

Spray PMS solution on 2.5 sq. cm portion of strip C on clean micro glass slide. Sandwich A and C strips between micro glass slides and place in 60°C hot air oven 5 min. Place small weight over slides for tight sandwich. (Warm with cigarette lighter flame under field conditions.) Mercury, copper, cadmium, and silver compounds appear as clear white spot against pink background.

Results and Discussion

The A strip impregnated with cholinesterase (ChE) enzyme present in the horse liver acetone powder metabolizes the 1-naphthyl acetate on strip B to naphthol which couples with Fast Blue B by diazotization to impart the magenta color. The appearance of a white spot on detector strip A, after reaction with strip B, represents the ChE inhibition caused by methyl parathion. Other organophosphorus compounds also inhibit this reaction, with various detection limits (Table 1). Sodium fluoride (400 ng) can also be detected.

The succinate dehydrogenase of strip A converts sodium succinate on strip C to fumarate in a dehydrogenation reaction (9); the electrons liberated are accepted by INT (10, 11) to develop a pink color due to the formation of formazan. PMS enhances this reaction (12). The appearance of a white inhibition spot in strip A after reacting with strip C represents succinate dehydrogenase inhibition caused by mercury, copper, cadmium, or silver compounds.

Table 1. Detection limits of detector strips for organophosphorus compounds and heavy metal compounds

Compound	Combination of reactive strips	Detection limits, μg
Copper sulfate	A + C	0.2
Cupric chloride	A + C	0.2
Mercuric chloride	A + C	0.1
Silver nitrate	A + C	0.2
Cadmium sulfate	A + C	1.0
Methyl paraoxon	A + B	0.1
Omethoate	A + B	0.1
Fenitrooxon	A + B	0.5

The combined A and B strips respond to oxygen analogs of organophosphorus compounds only. Organochlorines and other metal compounds (cobalt, nickel, lead, zinc, arsenic, antimony, strontium) do not interfere. The combined A and C strips do respond to copper, mercury, cadmium, and silver compounds, and therefore can only be used for rapid screening purposes (Table 1). Compounds must be qualitatively separated and identified by paper and thin layer chromatographic methods (8), or a modified method for better separation (in preparation). The prepared test sample should not contain acid or alkaline substances which cause enzyme denaturation. Heavy metal compounds can be detected directly in fresh or sea waters without cleanup, as also reported (8), whereas organophosphorus compounds can be extracted from water into hexane, concentrated after oxidation, and detected.

A solvent extract of water from an agricultural farm pond located at Pichattoor showed positive response to organophosphates on detector strips. In addition, 1% copper sulfate (Bordeaux) and mercury compounds used on seed and foliage at a horticulture unit were extracted in water from the surface deposits of samples and showed positive responses. It is suggested that for evaluation of heavy metals in fresh water, the water be evaporated to concentrate the pollutants to within detectable limits (Prameela Devi, Y., and Nanda Kumar, N. V., submitted to *J. Assoc. Off. Anal. Chem.*). Salt concentration has no effect on the color reaction (8). The combined A

and C strips do respond to barium chloride and to zinc chloride at 10 and 5 μg amounts, respectively.

Acknowledgment

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PRESERVATIVES

High Pressure Liquid Chromatographic Determination of Methyl and Propyl *p*-Hydroxybenzoates in Comminuted Meats

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A method was developed for determining methyl and propyl *p*-hydroxybenzoates (methyl and propyl parabens) in comminuted meats. The parabens were extracted from the meat sample with acetonitrile. After filtering, the extract was analyzed by reverse phase high pressure liquid chromatography, using a 254 nm absorbance detector. Samples of bologna, chicken roll, and chopped ham were fortified with approximately 100, 200, and 400 ppm of each paraben. Average recoveries were 92% for methyl paraben and 94% for propyl paraben.

Methyl and propyl parabens are effective inhibitors of *Clostridium botulinum* 10755A (1); therefore, it is likely that these substances will be considered as possible substitutes for nitrite salts in meats. Because this usage is not presently permitted, the Food and Drug Administration needs methodology to detect such unauthorized use of parabens. Thus it was our goal to develop a method for the determination of methyl and propyl parabens in comminuted meats.

Parabens in foods other than meat have been determined by thin layer chromatography (2–4), gas-liquid chromatography following conversion to the trimethylsilyl ethers (5–7), and anion exchange high pressure liquid chromatography (HPLC) (8). Methyl and propyl parabens have been determined in cosmetics by HPLC with ultraviolet (UV) detection at 254 nm (9). It was felt that determination of the parabens in comminuted meats by reverse phase HPLC with UV detection would offer the advantages of speed, simplicity, and selectivity not available with other techniques.

This paper describes a reverse phase HPLC method for the quantitation of methyl and propyl parabens in 3 different comminuted meats: bologna, chicken roll, and chopped ham. Recoveries from meat samples fortified with 100, 200, and 400 ppm of each paraben were determined. These levels were selected because studies (1) indicated that at least 200 ppm would be required to inhibit *C. botulinum* 10755A.

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METHOD

Reagents

(a) *Acetonitrile.*—Distilled-in-glass, UV grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442).

(b) *Water.*—Milli-Q water purification system (Millipore Corp., Bedford, MA 01730).

(c) *Methyl and propyl p-hydroxybenzoates.*—99% pure (Aldrich Chemical Co., Milwaukee, WI 53233).

Apparatus

(a) *Liquid chromatography.*—Spectra-Physics (Piscataway, NJ 08854) Model 3500B gradient liquid chromatograph equipped with Spectra-Physics Model 8200 absorbance detector set at 254 nm.

(b) *Injector.*—Micromeritics Model 725 automatic injector capable of injecting 10 μL aliquots (Micromeritics Instrument Corp., Norcross, GA 30093).

(c) *Strip chart recorder.*—Westronics MT 10 mV recorder (Tracor Westronics, Ft. Worth, TX 76106).

(d) *Column.*—4.6 mm id × 30 cm μBondapak C_{18}, reverse phase, 10 μm (Waters Associates, Milford, MA 01757).

(e) *Sample filtration apparatus.*—Stainless steel Swinny filter holder used with 1.0 μm Fluoropore filters (Millipore Corp.).

(f) *Blenders.*—Sorvall Omnimixer equipped with 500 mL cups (DuPont Co., Wilmington, DE 19898) for bologna and chicken roll; Cuisinart Model CFP9 food processor (Cuisinart Inc., Greenwich, CT 06820) for ham.

Standard Curve

Pipet 1.0, 2.0, 3.0, 4.0, and 5.0 mL methyl and propyl paraben solutions (500 μg/mL of each) into separate 100 mL volumetric flasks. Dilute to volume with acetonitrile, stopper, and mix carefully. This yields, respectively, standard concentrations of 5.0, 10.0, 15.0, 20.0, and 25.0 μg methyl and propyl parabens/mL.

Inject 10 μL aliquot of each standard solution

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onto HPLC column, using the following instrumental parameters: mobile phase: 45% (by volume) acetonitrile in water; flow rate: 1.4 mL/min; temperature: ambient. Select detector sensitivity of 0.16 absorbance unit full scale (AUFS) at 254 nm. Prepare standard curves by plotting peak heights vs concentration of standard solutions and drawing the best straight line through the points.

Extraction Procedure

Thoroughly grind ca 20 g meat sample, using Sorvall Omnimixer for bologna and chicken roll and Cuisinart Model CFP9 for ham. Weigh 2 g comminuted meat into 15 mL centrifuge tube equipped with screw cap. Add 5 mL acetonitrile to tube, cap tightly, and shake 30 s. Centrifuge, tightly capped, at low speed (ca 500 rpm) 5 min. Decant supernate into 25 mL volumetric flask. Repeat 3 times for bologna and chicken roll and 4 times for ham. Dilute to volume with acetonitrile. Filter aliquot through 1.0 μm Fluoropore filter for HPLC determinative step.

Determination

Inject 10 μL sample extract into chromatograph under conditions identical to those used to obtain standard curve data. Determine concentration of each paraben in sample extract using standard curve and peak heights for each paraben.

Calculate concentration of each paraben in sample as follows:

Paraben, ppm (μg/g) = $(C \times 25)/W$

where C = concentration, μg paraben/mL sample extract, 25 = volume (mL) of final extract, and W = weight (g) of meat sample extracted.

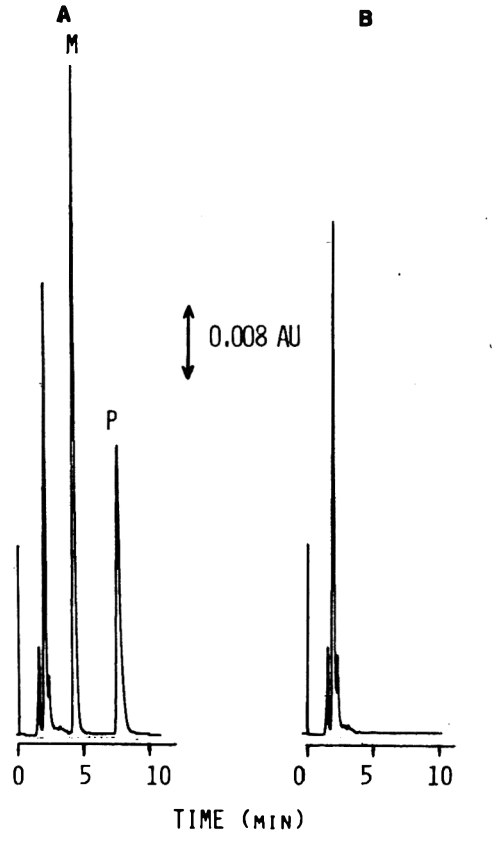

Figure 1. Chromatograms of bologna extracts. A, bologna containing 252 ppm methyl paraben and 222 ppm propyl paraben; B, unfortified meat. M, methyl paraben; P, propyl paraben. UV detector, wavelength 254 nm, sensitivity 0.16 AUFS.

Results and Discussion

Because methyl and propyl parabens are not now permitted in comminuted meats, no commercial food samples were available for analysis. It was therefore necessary to prepare fortified samples to use in developing and testing the method. To properly test the ability of the method to extract parabens from meat, it was essential that the spiking procedure yield a meat sample similar to that which would be produced commercially. To accomplish this, samples were fortified in the following manner: A portion of comminuted meat was accurately weighed, and known amounts of the 2 parabens were then added in solid form and thoroughly blended into the meat. In this manner, batches of bologna, chicken roll, and chopped ham containing 100, 200, and 400 ppm of each paraben were prepared.

Each sample was prepared for HPLC by extraction with acetonitrile followed by filtration to remove particulate matter. Acetonitrile was selected because it provides excellent recoveries of the parabens without extracting excessive amounts of fat. Typical chromatograms are shown in Figures 1–3. Retention times for methyl and propyl parabens were 4.2 and 7.6 min, respectively. Several unfortified chicken samples exhibited a small peak at the retention time of methyl paraben (Figure 2). This interference produced an error of about 1% at the 200 ppm level. The corresponding blanks for bologna and chopped ham (Figures 1 and 3) showed no interfering responses for methyl or propyl paraben.

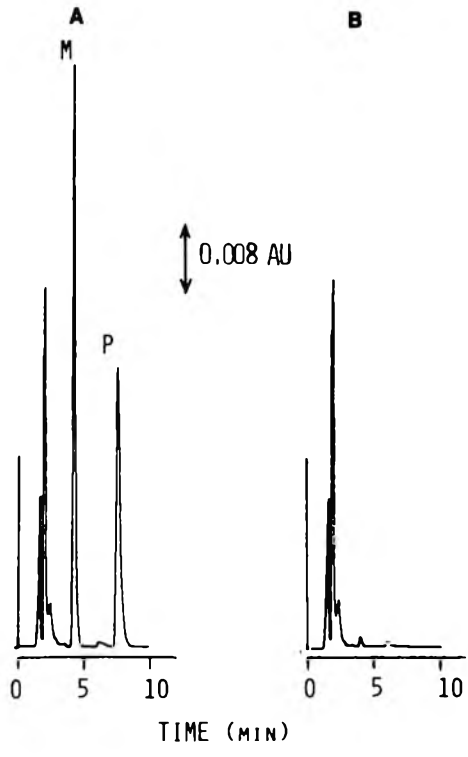

Figure 2. Chromatograms of chicken roll extracts. A, chicken containing 182 ppm methyl paraben and 224 ppm propyl paraben; B, unfortified meat. See Figure 1 caption.

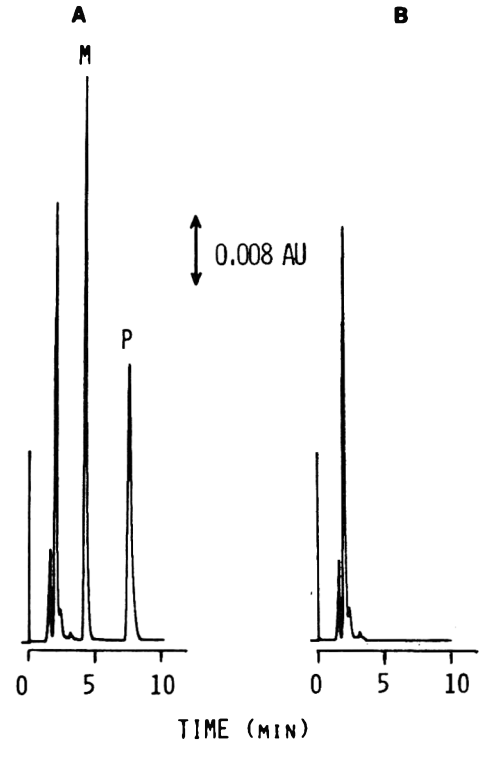

Figure 3. Chromatograms of chopped ham extracts. A, ham containing 229 ppm methyl paraben and 212 ppm propyl paraben; B, unfortified meat. See Figure 1 caption.

The recoveries for meat samples fortified with 100, 200, and 400 ppm of each paraben are summarized in Table 1. Eight to 10 replicate samples at each level of fortification were analyzed. The average recoveries were 92% for methyl paraben and 94% for propyl paraben with percent coefficients of variation of 2.2 and 2.4, respectively. The chromatograms in Figures 1–3 represent approximately 160 ng of each paraben injected on column.

The analytical method described in this work, using acetonitrile extraction coupled with HPLC, offers good separation of methyl and propyl parabens from components in comminuted

Table 1. Recoveries of methyl and propyl parabens from meats

Meat	Amount added, ppm		No. of samples	Av. rec., % (% CV)	
	Methyl	Propyl		Methyl	Propyl
Bologna	119	107	8	88.2 (0.54)	93.0 (1.20)
	252	222	10	91.1 (1.21)	94.5 (1.38)
	407	419	8	94.1 (0.50)	99.3 (0.88)
Chicken	118	113	10	91.5 (0.83)	92.1 (0.72)
	182	224	10	92.7 (0.49)	95.2 (0.84)
	414	420	9	94.1 (0.76)	95.8 (0.90)
Ham[a]	117	118	10	89.9 (0.61)	92.6 (0.54)
	229	212	9	89.7 (1.01)	92.6 (0.89)
	399	420	10	92.7 (0.79)	94.0 (1.10)

[a] Based on 4 extractions.

meats. Reliable quantitation can be achieved in the 100–300 ppm range typically used in foods (10) in which parabens are permitted.

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SYMPOSIUM

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DRUGS

Application of Chiral Lanthanide Nuclear Magnetic Resonance Shift Reagents to Pharmaceutical Analysis. II. Determination of Dextro- and Levoamphetamine Mixtures

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Optically pure *d*- and *l*-amphetamine sulfate, as well as various mixtures of the 2 enantiomers, were analyzed using a europium chiral nuclear magnetic resonance shift reagent. The enantiomeric shift difference ($\Delta\Delta\delta$) exhibited by the doublet associated with the α-methyl protons was large enough to differentiate between the *levo*- and *dextro*-isomers. The α-methyl protons were decoupled and the enantiomeric composition was determined by using the peak heights of the resulting singlets. As little as 5% of the *levo*-isomer in the presence of the *dextro*-isomer can be determined using this method. The method is applicable to the analysis of bulk drug and pharmaceutical preparations.

The derivatization of a drug substance, followed by the measurement of its specific rotation, is a common method for the determination of enantiomeric purity. The official method for the determination of the enantiomeric purity of *d*-amphetamine sulfate involves such a sequence: the preparation of acetylamphetamine, followed by purification and determination of the derivative's specific rotation (1). A similar, more sensitive assay has been developed, using 1-fluoro-2,4-dinitrobenzene as the derivatizing agent (2). While both of these methods give an accurate reflection of the enantiomeric composition, they do not directly quantitate the *dextro*- and *levo*-isomers. This measurement has been accomplished by gas-liquid chromatography of the *N*-trifluoroacetyl-*l*-proline derivatives of *d*- and *l*-amphetamine (3) and by nuclear magnetic resonance (NMR) analysis of the diastereomeric amides derived from *d,l*-amphetamine and (+)-α-methoxy-α-trifluoromethylphenyl acetic acid (4). All of these assays involve the derivatization of amphetamine and introduce the possibility of interferences from side reactions or impurities in the derivatizing agent.

The recent application of chiral lanthanide NMR shift reagents to the determination of the enantiomeric purity of isoquinoline alkaloids (5) and *d,l*-methorphan (6) makes possible the development of assays which do not involve the synthesis of derivatives. This paper reports the use of a europium chiral NMR shift reagent in the determination of the enantiomeric purity of amphetamine in bulk drug form and pharmaceutical preparations.

Experimental

Apparatus

A 200 MHz Fourier transform NMR spectrometer (Varian XL 200, Varian Instruments, Palo Alto, CA 94303) was used for all spectra.

Reagents

(a) *Deuterated benzene.*—Minimum isotopic purity 99.5 atom percent deuterium, used as purchased (Aldrich Chemical Co., Milwaukee, WI 53233).

(b) *Optically active europium chiral NMR shift reagent.*—Used as purchased (Alfa Products, Ventron Corp., Danvers, MA 01923). Before the start of each series of experiments, 0.2M stock solutions in carbon tetrachloride were prepared in a dry box under nitrogen atmosphere. Each solution was stored in a sealed vial fitted with a septum and sampled with a microliter syringe.

(c) *d-, l-, and d,l-Amphetamine sulfates.*—Used as purchased (K & K Laboratories, Plainview, NY 11803). Ten mg benzedrine sulfate tablets (Smith Kline & French) were purchased commercially.

Procedure

Bulk drug substance.—The appropriate amphetamine sulfate was dissolved in water, made basic with 10% NaOH, and extracted 3 times with ether. The ether extracts were combined and dried overnight using molecular sieves. Filtration and evaporation yielded the free base. Stock solutions (0.16M) of the free base were

[1] Food and Drug Administration Summer Graduate Intern, 1980.

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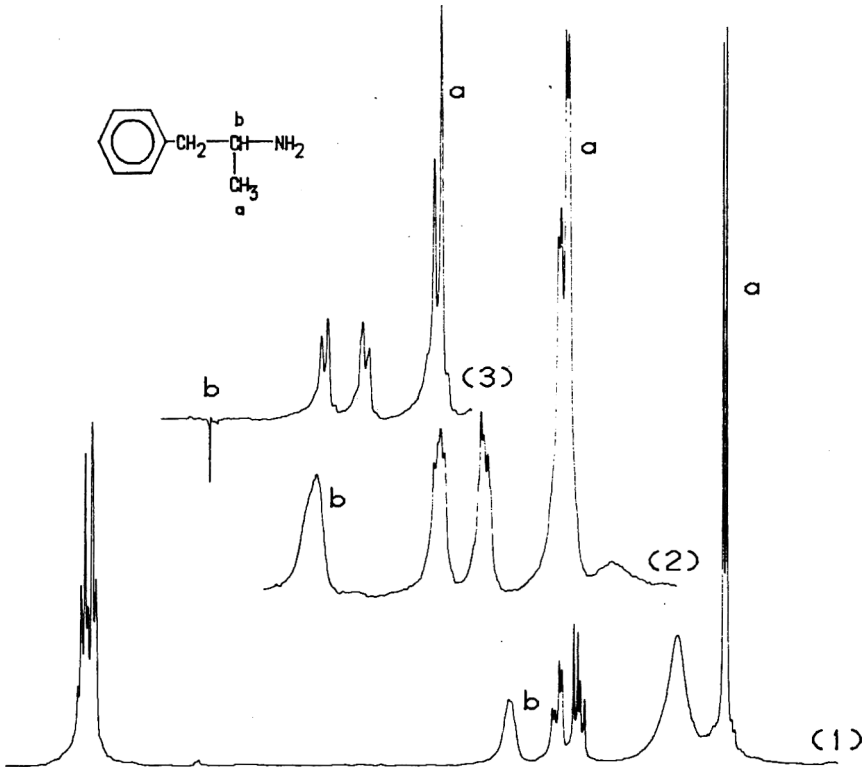

Figure 1. NMR spectra of a mixture by weight of 60% *d*-amphetamine-40%-*l*-amphetamine. (1), no shift reagent; (2), shift reagent to substrate molar ratio 0.15; and (3), spectrum (2) decoupled.

prepared by dissolving 65 mg of the respective enantiomer in 3.0 mL deuterated benzene. Enantiomeric mixtures were prepared from the stock solutions of the *dextro-* and *levo-*isomers.

The appropriate amphetamine solution, 0.40 mL, was placed in an NMR tube fitted with a rubber septum. The optimum molar ratio, 0.15, of shift reagent to substrate was determined by the incremental addition of 16 μL portions of the shift reagent solution to a mixture of *d-* and *l-*amphetamine (1 + 1). In these experiments, this ratio was produced by the addition of 48 μL shift reagent solution. In subsequent experiments, 48 μL shift reagent solution was added directly to the NMR tubes.

Tablet assay.—Twenty 10 mg capsules of benzedrine sulfate were ground to a fine powder. A portion equivalent to 20 mg amphetamine was transferred to a glass-stopper flask containing 5 mL 1N H_2SO_4. The resulting mixture was filtered through a Millipore filter (Type LS, 5 μm) and made basic with 50% NaOH. Then 0.50 g sodium sulfate was added to the solution followed by 0.80 mL hexadeuteriobenzene. The benzene layer was collected, dried over magnesium sulfate, and filtered through a glass wool-plugged pipet into an NMR tube.

Approximate concentrations of amphetamine in the solutions were determined by comparison of the peak height ratios of the proton of the pentadeuteriobenzene to the doublet of the amphetamine methyl protons in known reference solutions to those in the sample solution. This comparison is valid on Fourier transform NMR only if presaturation pulses are used or if a relatively large number of transients (at least 16) are taken. The sample solutions ranged in concentration from 0.10 to 0.12 M. As soon as the molar concentration was determined, the appropriate amount of shift reagent was added to obtain a 0.15 molar ratio of shift reagent to substrate.

Results and Discussion

The effect of the chiral europium NMR shift reagent on the NMR spectrum of a mixture of *l-* and *d-*amphetamine is shown in Figure 1. While both enantiomers displayed a noticeable induced

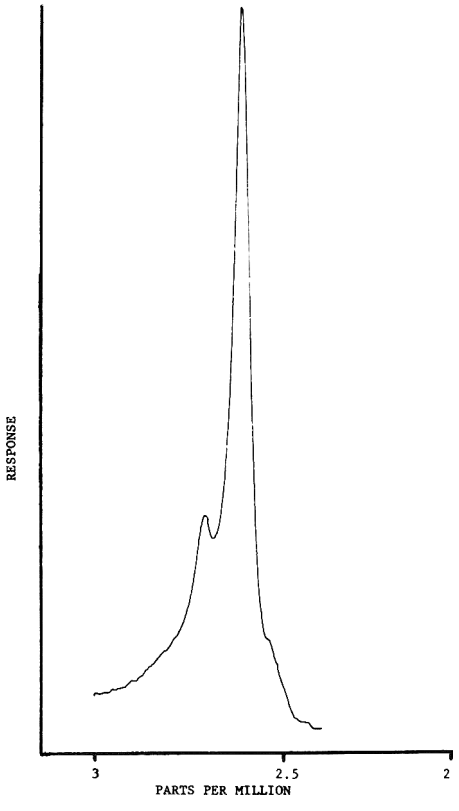

Figure 2. Decoupled spectrum of the α-methyl protons of a mixture of 95% *d*-amphetamine-5% *l*-amphetamine at a 0.15 molar ratio of shift reagent to substrate.

shift ($\Delta\delta$), the doublet associated with the α-methyl protons of the *levo*-isomer was shifted further downfield than the doublet associated with the *dextro*-isomer. This enantiomeric shift difference ($\Delta\Delta\delta$) was large enough to differentiate the *levo*- and *dextro*-isomers at a molar ratio of shift reagent to substrate as low as 0.05. Continued addition of the shift reagent increases the separation between the peaks but also broadens them, reducing the resolution. Optimum resolution was achieved at a 0.15 molar ratio of shift reagent to substrate.

The resolution was further enhanced by the irradiation of the absorption due to the methyne proton. This decouples the signal associated with the α-methyl protons, producing a pair of sharp singlets rather than a pair of doublets. In each individual determination, the exact shift of the absorption due to the methyne peaks had to be located, because its position varied widely with small changes in concentration. The effect of the decoupling on the NMR spectrum of a 60% dextroamphetamine-40% levoamphetamine mixture (by weight) is shown in Figure 1. This technique enabled the detection of as little as 5% *l*-amphetamine in the presence of *d*-amphetamine (Figure 2).

A series of enantiomeric mixtures ranging from 100% of the *levo*-isomer to 100% of the *dextro*-isomer was prepared from stock solutions of the respective free bases. A standard curve, constructed by comparing the molar fraction of the *dextro*-isomer determined from the peak heights of the decoupled NMR spectra (mole fraction *dextro*-isomer = peak height *dextro*-isomer + peak height *levo*-isomer) with the known molar fraction, was linear.

The versatility of this approach is demonstrated by its application to pharmaceutical preparations as well as the bulk drug. The results (molar fraction *d*-amphetamine) obtained from 4 assays, each carried out using the equivalent of two 10 mg tablets of a pharmaceutical preparation of racemic amphetamine, were 0.46, 0.46, 0.48, and 0.47 (mean 0.47 ± 0.01).

In conclusion, the europium chiral NMR reagent can be used to identify the enantiomers of amphetamine. This approach rapidly and accurately differentiates *l*-amphetamine and *d*-amphetamine and is a sensitive assay for the enantiomeric purity of both bulk drug and pharmaceutical preparations.

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High Pressure Liquid Chromatographic Determination of Sulfisoxazole in Pharmaceuticals and Separation Patterns of Other Sulfonamides

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A high pressure liquid chromatographic method has been developed for the identification and determination of sulfisoxazole in tablet, liquid, and ointment dosage forms. The method specifies a μBondapak C_{18} column, an acetonitrile–acetic acid–water (22.5 + 1 + 76.5) mobile phase, and ultraviolet detection at 254 nm. For quantitative purposes, sulfabenzamide is used as an internal standard. Commercial preparations were analyzed by the proposed method and results were compared with those obtained by the *U.S. Pharmacopeia* XIX and *National Formulary* XIV methods; good agreement was obtained. The elution behaviors of 19 additional sulfonamides were also established to verify the specificity of the proposed method for sulfisoxazole. These elutions patterns show potential for separation and identification of all 20 sulfonamides.

Sulfisoxazole [*N'*-(3,4-dimethyl-5-isooxazolyl) sulfanilamide] is a therapeutically useful antibacterial agent, commercially available in several dosage forms. Frequently this drug is found paired with diethanolamine under the designation of sulfisoxazole diolamine. Sulfisoxazole has been conventionally analyzed by nonaqueous titration (1, 2) and colorimetry (3). Both procedures are regarded as nonspecific and, in the latter case, time consuming as well.

High pressure liquid chromatography (HPLC) has been shown to be useful for the separation of sulfisoxazole from structurally related compounds. Using ion-exchange chromatography, Kram (4) studied the effects of mobile phase ionic strength on the elution times of 20 sulfonamides. Karger et al. (5) separated 12 sulfonamides on an in situ coated column by an ion-pair partition system, whereas Cooke et al. (6) separated 11 sulfonamides by using a mobile phase containing zinc ions chelated to 4-dodecyldiethylenetriamine (C_{12}-dien). Umagat et al. (7) were able to resolve a mixture of sulfonamides on a nitrile-bonded phase HPLC column by means of a nonaqueous mobile phase. Their method was shown to be applicable to the analysis of these compounds, including sulfisoxazole, in pharmaceutical dosage forms. More recently, Suber and Edds (8) reported on the HPLC separation of 8 sulfonamides with mobile phases at 2 different pH values.

This paper describes a method for the determination of sulfisoxazole in solid and liquid dosage forms, using reverse phase ion-suppression HPLC and ultraviolet (UV) detection at 254 nm. The order of elution of 19 additional sulfonamides on 2 HPLC columns with 3 different mobile phases is also presented, and shows potential as a rapid and satisfactory method of identification of all 20 sulfonamides in dosage forms. The method is stability indicating, is more convenient than nonaqueous titration, and avoids the use of dimethylformamide.

METHODS

Apparatus and Reagents

(a) *Liquid chromatograph.*—Du Pont 841 equipped with 254 nm UV detector (E. I. du Pont de Nemours and Co., Wilmington, DE 19898) and a 10 μL injection valve (Valco Instruments Inc., Houston, TX 77024).

(b) *Column.*—μBondapak® C_{18}, 300 × 3.9 mm (Waters Associates, Milford, MA 07157).

(c) *Data integration systems.*—Hewlett-Packard Model 3380-A (Hewlett-Packard, Avondale, PA 19311).

(d) *Mobile phase.*—Acetonitrile (Burdick & Jackson Laboratories, Muskegon, MI 49442, or equivalent)–acetic acid–double glass-distilled water (22.5 + 1 + 76.5).

(e) *Internal standard solution.*—Prepare a solution of sulfabenzamide (ICN Nutritional Biochemicals, Cleveland, OH 44128) in methanol to contain ca 1.0 mg/mL.

(f) *Reference standard preparation.*—Accurately weigh and quantitatively transfer ca 25 mg sulfisoxazole (USP Reference Standard, U.S. Pharmacopeial Convention, Rockville, MD 20852) to 100 mL volumetric flask, add 25.0 mL internal standard solution (e), and dilute to volume with

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methanol. Proceed as directed under *Determination*.

Preparation of Samples

Tablets.—Weigh and finely powder 20 tablets. Accurately weigh portion of powder to contain ca 500 mg sulfisoxazole, quantitatively transfer to 100 mL volumetric flask, add 25 mL methanol, shake mechanically for 30 min, dilute to volume with methanol, and filter. Transfer 5.0 mL filtrate to 100 mL volumetric flask containing 25.0 mL internal standard solution, mix, and dilute to volume with methanol. Proceed as directed under *Determination*.

Liquids (injections and ophthalmic solutions).—Accurately transfer volume of liquid dosage form containing ca 200 mg sulfisoxazole to 200 mL volumetric flask, and dilute to volume with methanol. Pipet 25.0 mL of this solution into 100 mL volumetric flask containing 25.0 mL internal standard solution, mix, and dilute to volume with methanol. Proceed as directed under *Determination*.

Ointments.—Accurately weigh portion of ointment to contain ca 50 mg sulfisoxazole, and quantitatively transfer to 125 mL separatory funnel containing 25 mL water–methanol (1 + 2) and 50 mL *n*-heptane. Shake, transfer lower layer to second 125 mL separatory funnel containing 50 mL *n*-heptane, shake, and transfer lower layer to 200 mL volumetric flask containing 50.0 mL internal standard solution. Extract *n*-heptane layers consecutively with three 25 mL portions of water–methanol (1 + 2), and combine these extracts with main extract in volumetric flask. Dilute to volume with methanol, and mix. Proceed as directed under *Determination*.

Determination

Equilibrate HPLC column with mobile phase at flow rate of 1.5 mL/min. Adjust mobile phase, if desired, by increasing acetonitrile concentration to decrease retention time of peaks at same flow rate. Retention time for sulfisoxazole should be between 6 and 10 min, and resolution, R (8), for sulfisoxazole and sulfabenzamide should be >2.0. Three replicate injections of the reference standard preparation (f) should give relative standard deviation of ratio of area of sulfisoxazole to area of internal standard <2.0%. When chromatographic conditions are met, inject sample solution for analysis.

Calculations

Calculate amount of sulfisoxazole by appropriate formula.

(a) Tablet: mg/tablet

$$= (R_{sm}/R_{st}) \times W_1 \times 20 \times (W_2/W_3)$$

(b) Liquid: mg/mL

$$= (R_{sm}/R_{st}) \times W_1 \times 8 \times (1/V)$$

(c) Ointment: mg/g

$$= (R_{sm}/R_{st}) \times W_1 \times 2 \times (1/W_3)$$

where R_{sm} = ratio of sulfisoxazole area to internal standard area in sample preparation; R_{st} = ratio of sulfisoxazole area to internal standard area in reference standard preparation; W_1 = weight in mg of sulfisoxazole in reference standard preparation; W_2 = average weight, g/tablet; W_3 = weight in g of sample taken; V = volume in mL of liquid dosage form taken (injection or ophthalmic solution).

Results and Discussion

The mobility behavior of sulfisoxazole and 19 other sulfonamides on 2 HPLC columns, using 3 mobile phase systems, is shown in Table 1. The separations reported in Table 1, Columns A

Table 1. Relative retention times [a] of sulfonamides

	Zorbax CN	μBondapak C_{18}	
Sulfonamide	A[b]	B[b]	C[b]
Sulfaquinoxaline	0.84	2.32	2.30
Sulfamethazine	0.91	2.93	0.40
Sulfadimethoxine	0.92	6.41	1.91
Sulfamethoxazole	0.99	1.81	0.78
Sulfisoxazole	1.00	1.00	1.00
Sulfaphenazole	1.01	1.29	1.86
Sulfamethoxypyridazine	1.02	2.55	0.47
Sulfapyridine	1.05	2.34	0.29
Sulfamerazine	1.08	3.96	0.32
Sulfacetamide	1.13	0.55	0.21
Sulfisomidine	1.18	1.32	0.26
Sulfadiazine	1.29	3.53	0.25
Sulfanilamide	1.33	0.55	0.14
Phthalylsulfathiazole	1.38	—[c]	1.48
Sulfathiazole	1.39	—[c]	0.29
Sulfaguanidine	1.54	0.52	0.13
Sulfachlorpyridazine	1.65	3.28	0.63
Sulfabenzamide	2.05	1.07	1.24
Sulfamethizole	2.18	5.19	0.47
Succinylsulfathiazole	ND[d]	—[c]	0.49
Solvent emergence, min	1.21	2.2	2.2
Sulfisoxazole emergence, min	7.53	6.2	20.8
Flow rate, mL/min	3.00	1.6	1.6

[a] Retention times are expressed relative to sulfisoxazole taken as 1.00.

[b] Mobile phase: A, methanol–methylene chloride–*n*-heptane (10 + 15 + 75); B, acetonitrile–water (35 + 65) containing 10^{-3}M $ZnSO_4$, 0.025% C_{12}-dien, 1% ammonium acetate; C, acetonitrile–acetic acid–water (12.5 + 1 + 86.5).

[c] Retained on column over 1.5 h.

[d] Not determined.

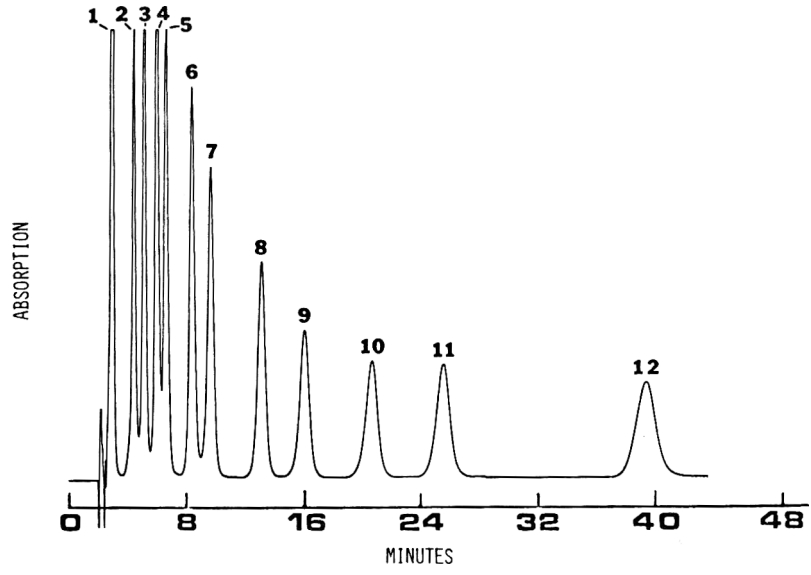

Figure 1. Chromatographic separation of sulfonamides. Column μBondapak C_{18}; mobile phase acetonitrile-acetic acid-water (12.5 + 1 + 86.5); pressure at inlet 1300 psig; flow rate 1.6 mL/min; sensitivity 0.16 absorbance unit full scale (AUFS). 1, sulfanilamide; 2, sulfacetamide; 3, sulfadiazine; 4, sulfapyridine; 5, sulfamerazine; 6, sulfamethazine; 7, sulfamethizole; 8, sulfachlorpyridazine; 9, sulfamethoxazole; 10, sulfisoxazole; 11, sulfabenzamide; 12, sulfadimethoxine.

and B, are extensions of work by Umagat et al. (7) and Cooke et al. (6), respectively. Although both separations were useful, some shortcomings were noted during the analysis of sulfisoxazole.

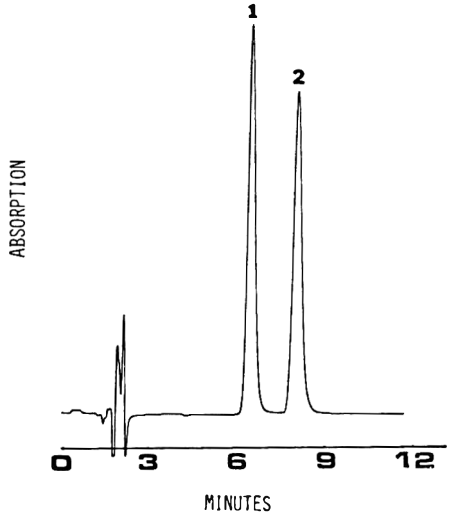

Figure 2. Chromatographic separation of sulfisoxazole and sulfabenzamide. Column μBondapak C_{18}; mobile phase acetonitrile-acetic acid-water (22.5 + 1 + 76.5); pressure at inlet 1500 psig; flow rate 1.5 mL/min; sensitivity 0.32 AUFS. 1, sulfisoxazole; 2, sulfabenzamide.

For example, the normal phase system of Umagat et al. (7) on the Zorbax CN column used in this study was unable to separate sulfisoxazole from several other sulfonamides, especially sulfamethoxazole and sulfaphenazole possibly because of differences in column characteristics. In addition, this approach required sulfisoxazole to be in an organic solvent, so that it was less directly applicable to aqueous dosage forms. Although the reverse phase system of Cooke et al. (6), using a mobile phase containing zinc ions, achieved greater resolution of sulfisoxazole and other related sulfonamides, it required a long equilibration time and the removal of the zinc ions at the end of the chromatographic analysis. The proposed HPLC separation (Table 1, Column C) differs from that of Cooke et al. in that the mobile phase consists of a mixture of acetonitrile-acetic acid-water. This modification yields improved resolutions and metal-free conditions.

Figure 1 illustrates a typical separation of sulfisoxazole from 11 other sulfonamides, a number of which are listed in the *U.S. Pharmacopeia* XIX and *National Formulary* XIV. By increasing the content of acetonitrile in the mobile phase, retention times could be shortened without any apparent changes in the order of elution of the various sulfonamides. The mobile phase used in the determination of sulfisoxazole in phar-

Table 2. Analysis of sulfisoxazole and sulfisoxazole diolamine in commercial preparations

Sample	Declared	Found, % of declared	
		Liquid chromatography	Compendial method
Sulfisoxazole tablets 1	0.5 g/tab	100.6, 101.4	101.0, 102.6[a]
Sulfisoxazole tablets 2	0.5 g/tab	95.6, 95.6	95.8, 95.8[a]
Sulfisoxazole tablets 3	0.5 g/tab	99.8, 99.7	99.6, 100.1[a]
Sulfisoxazole diolamine injection	40 mg/mL[b,c]	101.2, 100.9	99.3, 99.1[d]
Sulfisoxazole diolamine ophthalmic solution	40 mg/mL[c]	104.5, 104.7	103.2, 102.2[d]
Sulfisoxazole diolamine ophthalmic ointment	40 mg/g[c]	106.0, 107.8	108.0, 107.8[d]

[a] USP XIX.
[b] Synthetic preparation.
[c] As sulfisoxazole.
[d] NF XIV.

maceuticals was modified accordingly to reduce the assay time. Because of its distinct separation from sulfisoxazole, sulfabenzamide was chosen as the internal standard (Figure 2).

The relationship between the response of the detector and the amount of sulfisoxazole injected was linear over the range 0.0–3.5 μg of drug injected. Although differences were noted in the retention of these compounds on each of 5 brands of columns, their relative retention values with respect to sulfisoxazole were in agreement with those observed on the μBondapak C_{18} column.

Table 2 summarizes the results of the assays of tablet, liquid, and ointment dosage forms by the proposed HPLC method and by compendial methods. In all cases, results of replicate assays of the same sample differed from one another, on the average, by less than 2%. Good agreement was also found between results obtained by proposed and by compendial methods. Because a commercial sample of sulfisoxazole diolamine injection was unavailable, a synthetic one was prepared and analyzed along with the other samples. The recovery value for this sample was 101.1%.

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Spectrophotometric Determination of Atropine Sulfate in the Presence of Phenylmercury (II) Acetate

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Two-component spectrophotometric method of analysis using 2 wavelengths, the method of least squares using absorbances, the method using 2-orthogonal function coefficients, and the method of least squares using orthogonal function coefficients have been applied to the determination of atropine sulfate in the presence of phenylmercury (II) acetate, compounds whose spectra overlap. The first method gave erroneous results; the second method gave satisfactory results for synthetic mixtures. The fourth method was superior, especially in the presence of irrelevant absorption. It has been successfully used for determining atropine sulfate in injection solutions in which a cubic irrelevant absorption was present. Results were in good agreement with those obtained by the official method.

When properly applied, the 2-wavelength method of analysis (1) gives excellent results for the analysis of a mixture of 2 absorbing compounds if the 2 spectra are sufficiently separated and if irrelevant absorption is completely absent. In practice, however, certain groups of compounds may possess similar and overlapping spectra. This is true for atropine sulfate and phenylmercury (II) acetate (Figure 1). In such a case, the use of the modified Vierordt method (2) would give erroneous results.

To solve this problem, several authors introduced the use of least squares to improve assay precision and accuracy. Cama et al. (3) used 16 wavelengths to assay vitamin A in cod liver oil. Neuer (4) discussed the mathematical basis of multicomponent spectrophotometric analysis using least squares. Klabuhn et al. (5) used 200 wavelengths to analyze a mixture of 4 compounds. Madsen et al. (6, 7) used least squares after the Gaussian expansion of absorption spectra in the analysis of pharmaceutical mixtures. Wahbi et al. (8) used both absorbances and orthogonal function coefficients under least squares for 3-component analysis.

Unterhalt and Wahbi (9) proved theoretically that the greater the number of points used in the calculation process, the greater the precision of an assay. Wahbi et al. (10) used least squares to determine the minor component in 2-component spectrophotometry. However, it should be emphasized that the method of least squares using absorbances eliminates random error; irrelevant absorption if present, would also lead to erroneous results. This situation imposes restrictions on the use of least squares in spectrophotometric analysis because interferences may arise from batch-to-batch differences or from general contamination of solutions during their preparation. In this connection, Glenn (11) proposed the use of orthogonal functions to eliminate interferences in 2-component analysis.

Glenn's method of orthogonal functions has been successfully applied to the determination of a single substance in the presence of interferences, using a suitable single coefficient (12–14). In 2-component analysis, 2 coefficients of the same order calculated at 2 different sets of wavelengths or 2 coefficients of different order calculated at one set of wavelengths can be used.

Development of Method

Two-Component Spectrophotometry Using Two Wavelengths

Seven mixtures of atropine sulfate and phenylmercury (II) acetate in 0.1N H_2SO_4 were prepared and atropine sulfate was determined at 2 absorbances, 252 and 257 nm. Percent recoveries were recorded between 45.4 and 127.5 (Table 1). These results were not surprising because the spectra of the 2 compounds are greatly overlapping; the ratio of absorbance ratios defined by Glenn (2) was within the exclusion range.

Least Squares Applied to Two-Component Spectrophotometry

To improve the results, the method of least squares was applied to determining atropine sulfate in the presence of phenylmercury (II) acetate. Thus, using 35 absorbances measured at 1 nm intervals from 242 to 276 nm, the mean percent recovery was 100.4 ± 0.92 (Table 1).

Use of Orthogonal Functions to Eliminate Interferences

According to general rules collated by Wahbi et al. (12, 13) the quartic and quintic polynomials

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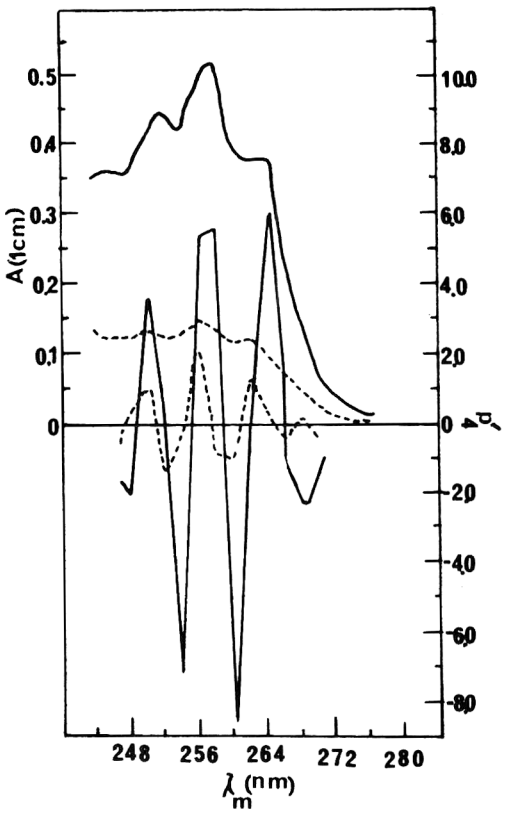

Figure 1. Absorption curves for 0.1% atropine sulfate (—), 0.02% phenylmercury (II) acetate (- - -), and their corresponding p'_4 convoluted curves.

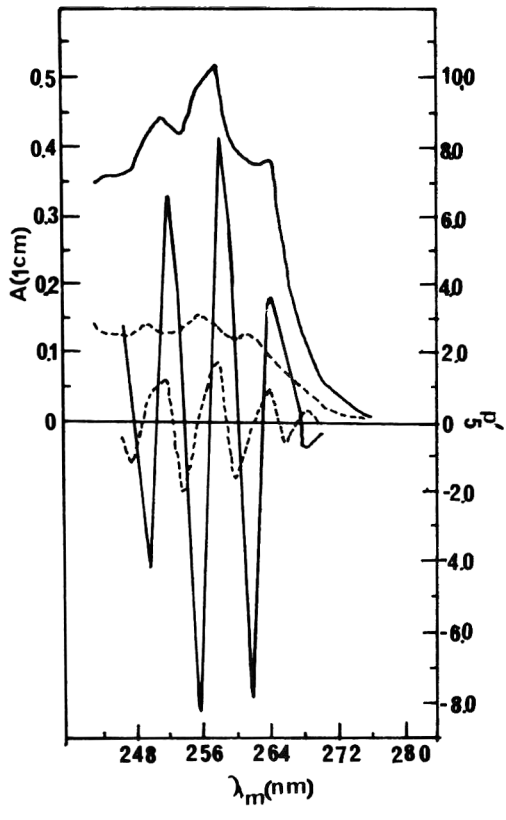

Figure 2. Absorption curves for 0.1% atropine sulfate (—), 0.02% phenylmercury (II) acetate (- - -), and their corresponding p'_5 convoluted curves.

(P_4 and P_5, respectively) make large contributions to the absorption spectra of atropine sulfate and phenylmercury (II) acetate in 0.1N H_2SO_4 (Figures 1 and 2). Accordingly, the coefficients p_4 and p_5 would afford precise estimates of concentration.

Twelve-point orthogonal polynomials were used in the present work because the irrelevant absorption curve present in atropine sulfate injection solutions proved to be cubic in nature (Figure 3). The optimum sets of wavelengths were chosen by plotting p_4 and p_5 calculated at 1 nm intervals against λ_m, the mean of the set of wavelengths, a process that leads to the convoluted absorption curves (15). The optimum wavelength ranges were selected to maximize p_4 or p_5 whenever relevant (Figures 1 and 2).

The results obtained for determining atropine sulfate in the presence of phenylmercury (II) acetate, using p_4 calculated at λ_{m1} = 254.5 nm and λ_{m2} = 260.5 nm at 1 nm intervals and using p_5 calculated at λ_{m1} = 256.5 nm and λ_{m2} = 258.5 nm, are shown in Table 1. Mean percent recoveries were 101.6 ± 2.0 and 101.9 ± 1.2, respectively. These results are highly precise and accurate when compared with those obtained using 2 absorbances. The calculation of p_4 (or p_5) using 12-point orthogonal polynomials rejects all components from p_0P_0 to $p_{11}P_{11}$ inclusive in the reference and mixture absorption curves and hence leads to better resolution. The *F*- and *t*-tests did not reveal any significant difference between the results obtained using p_4 or p_5 with regard to precision and accuracy. The calculated and theoretical *F*-values were 2.66 and 4.28, respectively. The calculated and theoretical *t*-values were 0.34 and 2.18, respectively at P = 0.95.

Least Squares Applied to Orthogonal Function Method

To further improve these results, we applied the orthogonal function method under least

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Table 1. Spectrophotometric determination (%) of atropine sulfate in the presence of phenylmercury (II) acetate by 4 procedures

		Absorbance		Orthogonal function			
				2 Coeff.		Least sq., 24 coeff.[e]	
Soln[a]	Atrop. sulf., g%	2 Wavelengths, 252 and 257 nm	Least sq., 242–276 nm[b]	p_4^c	p_5^g	p_4	p_5
1	0.100	127.5	99.5	97.3	100.6	101.8	102.2
2	0.150	88.4	99.8	101.1	103.8	103.3	102.2
3	0.080	92.7	99.6	102.8	100.7	100.5	101.4
4	0.120	69.2	101.1	102.8	103.2	103.2	103.5
5	0.100	45.4	102.0	102.7	101.6	101.9	101.8
6	0.150	69.8	100.2	102.0	101.4	101.9	100.9
7	0.080	75.7	100.6	102.5	102.3	101.5	101.3
Mean ± SD			100.4± 0.92	101.6± 1.99	101.9± 1.22	102.0± 0.97	101.9± 0.85
$F(0.95)$				2.66(4.28)		1.30(4.28)	
$t(0.95)$				0.34(2.18)		0.20(2.18)	

[a] Laboratory-prepared solution; each contains 0.010% phenylmercury (II) acetate.
[b] 35 absorbances measured at 1 nm intervals.
[c] λ_{m1} and λ_{m2} = 254.5 and 260.5 nm, respectively.
[d] λ_{m1} and λ_{m2} = 256.5 and 258.5 nm, respectively.
[e] Coefficients calculated at 1 nm intervals from λ_{m1} = 247.5 nm to λ_{m24} = 270.5 nm.

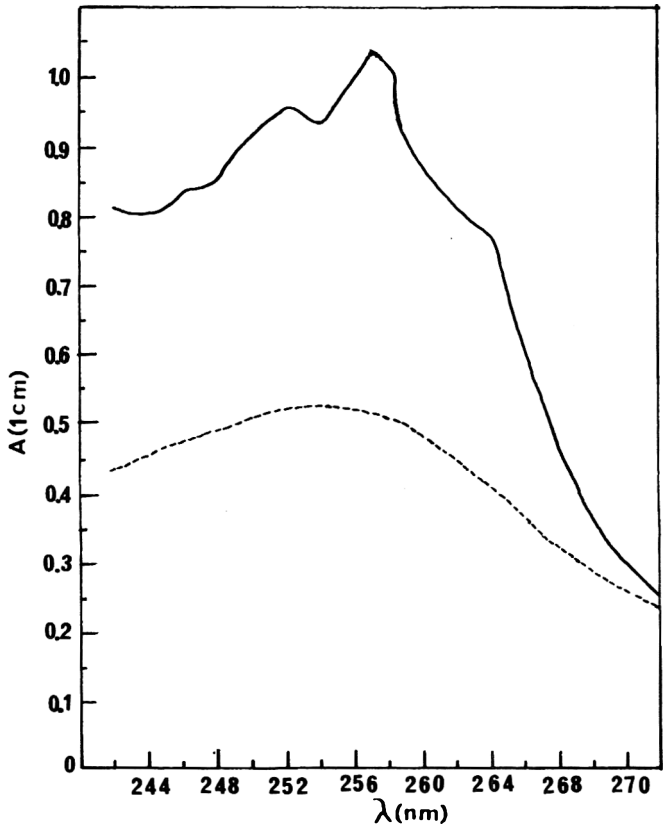

Figure 3. Absorption curve for atropine sulfate injection solution (—) labeled 1 mg/mL and the associated irrelevant absorption curve (- - -) obtained using the compensation method.

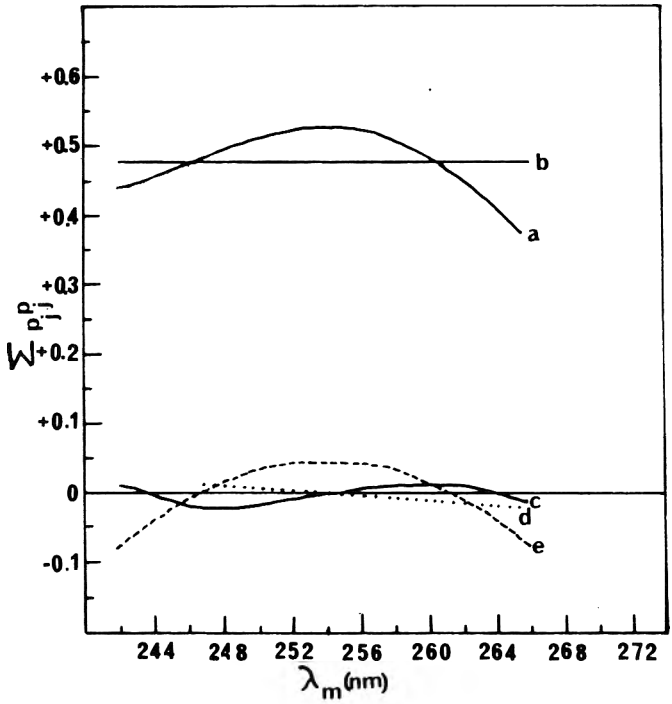

Figure 4. Expansion of the irrelevant absorption curve present in atropine sulfate injection solution in terms of orthogonal functions using 24 points. a = irrelevant absorption, b = p_0P_0, c = p_3P_3, d = p_1P_1, e = p_2P_2.

squares. Thus, 24 coefficients (p_4 or p_5) starting from λ_{m1} = 247.5 nm to λ_{m24} = 270.5 nm, at 1 nm intervals for reference solutions of atropine sulfate (0.1%), phenylmercury (II) acetate (0.020%), and the mixtures have been calculated. The concentration of atropine sulfate in each mixture was computed using the method of least squares. Mean percent recoveries were 102.0 ± 0.97 using p_4, and 101.9 ± 0.85 using p_5. These results are more precise than those obtained using 2 coefficients (Table 1). *F*- and t-tests showed no significant differences between the results obtained using p_4 or p_5 under least squares. The calculated and theoretical *F*-values were 1.30 and 4.28, respectively; calculated and theoretical *t*-values were 0.20 and 2.18, respectively, at P = 0.95.

Determination of Atropine Sulfate in Injection Solutions

Injection solutions of atropine sulfate are usually prepared to contain 0.1% atropine sulfate with 0.003% phenylmercury (II) acetate added as a bactericide (16). The acidity of the aqueous solution is adjusted to pH 3 with dilute H_2SO_4. Several methods have been reported for determining atropine sulfate in injections, including spectrophotometric (17), fluorometric (18), titrimetric (19), colorimetric (20–23), and gas-liquid chromatographic methods (24, 25). In view of the low absorptivities of atropine sulfate (a = 0.5 at 252 nm, 0.6 at 258 nm, and 0.5 at 264 nm, where a = absorbance of 1 g/L, 1 cm), its absorption curve in injection solutions is usually contaminated by the reagents used. The shape of the irrelevant absorption curve has been revealed using the compensation technique, as shown in Figure 3 for an injection solution. This curve has been expanded in terms of orthogonal functions over the wavelength range 242–266 nm at 1 nm intervals (Figure 4), where an equation of the form $f(\lambda) = 0.479P_0 + 0.00196P_1 - 0.000841P_2 + 0.0000240P_3$ represents the best fit. This indicates that the irrelevant absorption curve is cubic in nature. For this reason, the quartic, P_4, and quintic, P_5, polynomials were chosen to determine atropine sulfate in injection solutions. We applied the orthogonal function methods using 2 coefficients and also using 24 coefficients under least squares to the determination of atropine sulfate in 4 commercial samples for injection.

Table 2. Spectrophotometric determination of atropine sulfate (mg/mL) in injection solutions [a] by 3 procedures

	2 Coeff.		Least sq., 24 coeff.		
Sample	p_4	p_5	p_4	p_5	Official
A	0.985	0.990	0.988	0.991	0.988
B	1.010	1.008	1.005	1.007	1.009
C	1.007	1.009	1.003	1.010	1.000
D	0.971	0.970	0.983	0.980	0.975

[a] Commercial solutions, 1 mg atropine sulfate/mL.

Experimental

Apparatus

Spectrophotometer.—Single beam, manual (Prolabo, Jean & Constant, Paris, France).

Procedure

Measure absorbances for 1 cm cell path of injection solutions over wavelength range 242 to 276 nm each at 1 nm intervals. Similarly measure a reference solution of atropine sulfate (0.100% in 0.1N H_2SO_4) and a reference solution of phenylmercury (II) acetate (0.100% in 0.1N H_2SO_4). Calculate (26) the quartic coefficient, p_4, from the expression $[A_0(+33) + A_1(-27) + A_2(-33) + A_3(-13) + A_4(+12) + A_5(+28) + A_6(+28) + A_7(+12) + A_8(-13) + A_9(-33) + A_{10}(-27) + A_{11}(+33)]/8008$. Calculate the quintic coefficient, p_5, from the expression $[A_0(+33) + A_1(-57) + A_2(-21) + A_3(+29) + A_4(+44) + A_5(+20) + A_6(-20) + A_7(-44) + A_8(-29) + A_9(+21) + A_{10}(+57) + A_{11}(-33)]/15912$.

The subscripts 0, 1, 2, . . . , 11 represent λ_0, λ_1, λ_2, . . . , λ_{11}, respectively.

Results and Discussion

Four different commercial atropine sulfate injection solutions were assayed using the different methods. The results obtained using (*1*) 2 coefficients, (*2*) 24 coefficients under least squares, and (*3*) the official method agreed well (Table 2). The improved results obtained using the orthogonal function methods (p_4 or p_5 whenever relevant) were not surprising because the irrelevant absorption present has been proved to be cubic and therefore its contribution to either the quartic or quintic coefficients is very negligible.

The present work offers general methods for the analysis of binary mixtures with overlapping spectra in the presence of irrelevant absorption. The latter should differ in shape and nature from the compounds to be investigated.

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Bromination Methods for Determination of Phenylephrine Hydrochloride in Nose Drops, Salicylamide in Tablets, and Tetracycline Hydrochloride in Capsules

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Two methods, kinetic and titrimetric, based on an electrophilic substitution reaction with bromine, are described for the assay of phenylephrine hydrochloride in nose drops, salicylamide in tablets, and tetracycline hydrochloride in capsules. The kinetic method depends on the linear relationship between concentration of the drug (μg/mL) and time (s) for nuclear monobromination, indicated by bleaching of the methyl orange acid color. The titrimetric method is based on addition of excess of bromate-bromide with subsequent determination of excess bromine by an iodide-thiosulfate procedure. This method is also used to count the number of bromine atoms necessary for the electrophilic substitution reaction. Preservatives or tablet/capsule bases do not interfere in either method. Recoveries of drugs added to laboratory-prepared samples were good. As indicated by the *F*-test, the methods are equally reproducible.

The sympathomimetic phenylephrine hydrochloride, the analgesic and antipyretic salicylamide, and the antibiotic tetracycline hydrochloride are widely used in pharmaceutical practice. Phenylephrine has been assayed either in unit doses or in combination with other drugs by colorimetry (1, 2), ultraviolet (UV) spectrophotometry (3–5), chromatography (6, 7), and nonaqueous titrimetry (8). Methods described for determining salicylamide include colorimetry (9, 10), UV spectrophotometry (11–13), and differentiating nonaqueous titrimetry (14–16). Tetracycline has been determined by colorimetry (17, 18), UV spectrophotometry (19, 20), fluorometry (21–24), thin layer chromatography (25, 26), column chromatography-absorption spectrophotometry (27), high performance liquid chromatography (28), and densitometry (29, 30).

Bromine solution has been used in analytical determinations for some time. The most widely used brominating agent is bromate-bromide solution, first proposed by Francis (31). The bromine generated on addition of acid is used to brominate phenylephrine, salicylamide, and tetracycline molecules. The kinetic method described here is based on the linear relationship between bromination time and concentration of drug. The titrimetric method, described in the USP assay of phenylephrine hydrochloride powder (32), is based on addition of excess bromate-bromide solution with subsequent determination of the excess bromine by using an iodide-thiosulfate procedure. This titrimetric method is applied here for assaying salicylamide and tetracycline. The method is also used to count the bromine atoms necessary for bromination of the drugs under investigation.

METHODS

Reagents and Materials

(a) *Standard solutions.*—(*1*) Phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, MO 63178), 0.1% in water. (2) Salicylamide (Merck), 0.5%. Dissolve in 8 mL 40% ethanol and dilute to 100 mL with water. (3) Tetracycline hydrochloride (Pharchem Industries (Nigeria Ltd) Lagos, Nigeria), 0.1% in water.

(b) *Reagents.*—(*1*) Methyl orange solution: Dissolve 40 mg methyl orange in 1 L 0.3N H_2SO_4. (*2*) 0.1M Potassium bromate–0.2M potassium bromide mixture for kinetic method. (*3*) 0.1N Potassium bromate–0.1N potassium bromide mixture for titrimetric method. (*4*) 0.1N Sodium thiosulfate. (5) Potassium iodide. All reagents are Merck products used as received.

(c) *Tablet/capsule excipients.*—Chlorobutanol (British Drug House), and lactose, starch, talc, and magnesium stearate (Merck). All analytical grade and used as received.

Preparation of Assay Solutions

Phenylephrine nose drops.—Pipet 10 mL (equivalent to 0.05 g phenylephrine hydrochloride) of solution into 50 mL volumetric flask and dilute to volume with water. Accurately measure ca 10 mL (for kinetic method) or 25 mL (for titrimetric method) and proceed as described below.

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Salicylamide tablets.—Transfer 1 tablet (equivalent to 0.5 g salicylamide) to 100 mL volumetric flask, and add 8 mL of 40% ethanol. Shake until tablet disintegrates. Add 40 mL water, and shake 10 min. Dilute to volume with water. Accurately measure ca 10 mL (for kinetic method) or 5 mL (for titrimetric method) and proceed as described below.

Tetracycline capsules.—Transfer contents of 1 capsule (equivalent to 0.25 g tetracycline hydrochloride) to 100 mL volumetric flask. Add ca 20 mL water and dissolve as completely as possible by shaking. Dilute to volume with water. Accurately measure 10 mL (for kinetic method) or 15 mL (for titrimetric method) and proceed.

Procedures

Kinetic method.—Transfer appropriate aliquot of assay solution (see above) to 50 mL volumetric flask containing 25.0 mL methyl orange solution. Dilute to volume with water. Into separate test tubes of similar dimensions, measure 5.0 mL prepared solution and 5.0 mL 0.1M bromate–0.2M bromide mixture. Immerse both tubes in ice bath until they reach 4–5°C. Start stopclock (accurate to 0.2 s) and thoroughly mix the 2 solutions, noting time of addition (initial time, T_i). Stir mixture gently with thermometer used for temperature measurement. Note time required for methyl orange color to discharge (final time, T_f). Compute actual time (T_e), where $T_e = T_f - T_i$. Carry out blank experiment simultaneously by mixing bromate-bromide mixture with methyl orange solution, omitting addition of drug. Bleaching time for blank experiment is T_b and equals ~7 s. Compute corrected time (T_c), where $T_c = T_e - T_b$. Use regression equations 1, 2, and 3 for concentration calculation.

$$T_c = \text{intercept} + (\text{slope} \times \text{concentration})$$

Titrimetric method.—Transfer appropriate aliquot of assay solution (see above) to iodine flask. Dilute to ca 25 mL with water. Add 50.0 mL 0.1N bromate-bromide mixture followed by 5 mL of concentrated HCl. Keep flask out of direct light for 15 min (phenylephrine and salicylamide) or 30 min (tetracycline), and shake at intervals during this time. Keep flasks in dark an additional 15 min in all cases. Add ca 1.5 g potassium iodide and wash stopper and flask neck with 10 mL water. Add 5 mL $CHCl_3$, and titrate solution with 0.1N sodium thiosulfate to straw yellow color. Add a few drops of starch solution and continue titration with vigorous shaking until color discharge of bluish aqueous layer and violet $CHCl_3$ layer. Conduct blank experiment simultaneously. Calculate volume (V) of 0.1N bromate-bromide solution by subtracting sample from blank reading. Multiply V by 3.3901, 2.2395, or 5.0316 mg to calculate amount of phenylephrine hydrochloride, salicylamide, or tetracycline hydrochloride, respectively.

Results and Discussion

The phenolic group in the molecule of the drugs under investigation acts as an electron donor. This group increases the electron density of the ring by virtue of its inductive-resonance effect on the electron distribution, accounting for the observed activation at the ortho and para positions where the electron density reaches a maximum. The net result of the electrophilic substitution reaction is, therefore, the introduction of bromine, generated on addition of acid to bromate-bromide mixture, at the ortho and/or para position(s) to the phenolic group in the drug molecule.

Rationalization of mononuclear bromination in the kinetic assay applies the concept of reaction rates. The dibromination rate constant for phenol is much lower than the monobromination rate constant. The latter is reported to be 6.5 $\times 10^6$ L mol^{-1} s^{-1} (33), much faster than the rate of bromine production following addition of acid to bromate-bromide mixture at 25°C (34). However, using small concentrations of the drug and decreasing the reaction temperature to 4–5°C, a small steady state concentration of bromine, which acts as a chemical clock, is set up at a rate that approximates the monobromination rate. The sharp increase in bromine production after monobromination is sufficient to discharge the acid color of methyl orange, and the time of bleaching is directly proportional to the concentration of the drug. Extrapolation of this linear relationship intercepts the time axis at a point almost equal to the blank reading. The experiment reading can therefore be corrected. The calibration curves of time (T_c in seconds) vs concentration (C), in the range 100–500 μg/mL (phenylephrine and tetracycline hydrochlorides) or 500–2500 μg/mL (salicylamide) calculated in the final dilution after methyl orange addition, can be described by the following regression equations derived by the method of least squares (35).

$$T_c = -2.0550 + 0.0997C, \text{ for phenylephrine} \quad (1)$$

$$T_c = -2.8850 + 0.0181C, \text{ for salicylamide} \quad (2)$$

$$T_c = -1.0000 + 0.0600C, \text{ for tetracycline} \quad (3)$$

Table 1. Recovery of drugs from laboratory-prepared simulations

Drug[a]	Kinetic method: Added, g(range)	Kinetic method: Mean % rec ± SD (*n*)[b]	*F* value	Titrimetric method: Added, g(range)	Titrimetric method: Mean % rec. ± SD (*n*)
Phenylephrine	0.010–0.020	99.81 ± 0.71 (6) (0.65)[c]	0.73[d]	0.015–0.025	100.37 ± 0.85 (5) (0.98)
Salicylamide	0.080–0.160	101.49 ± 1.46 (6) (2.50)	3.42[e]	0.020–0.040	100.68 ± 0.79 (6) (2.11)
Tetracycline	0.025–0.100	100.5 ± 1.00 (6) (1.10)	2.312[f]	0.030–0.050	99.97 ± 0.71 (4) (0.08)

[a] (*1*) For phenylephrine, prepare by dissolving 0.5 g phenylephrine hydrochloride and 0.5 g chlorobutanol in 100 mL water. (*2*) For salicylamide, prepare by dissolving 0.5 g salicylamide and 0.5 g lactose as described for salicylamide tablets in Preparation of Assay Solutions. 0.1 g starch, 0.1 g talc, and 0.1 g magnesium stearate were added before dilution to 100 mL. (*3*) For tetracycline, prepare by dissolving 0.25 g tetracycline hydrochloride and 0.5 g lactose in 100 mL water.

[b] *n* = number of experiments; SD = standard deviation.

[c] Values in parentheses are the calculated *t* values for which theoretical t_0 0.975 equals 2.571 (for *n* = 6), 2.776 (for *n* = 5), or 3.182 (for *n* = 4).

[d] Calculated *F* value for which *F* theoretical at 5% level is 5.19.

[e] Calculated *F* value for which *F* theoretical at 5% level is 5.05.

[f] Calculated *F* value for which *F* theoretical at 5% level is 9.01.

The regression coefficients for the above relationships were calculated to be 0.9999 (Eq. *1*), 0.9990 (Eq. 2), and 1.0000 (Eq. 3). Restandardization of the above calibration curves was occasionally checked.

Because the inductive effect of the phenolic group is decreased by distance, substitution by the bromine electrophile in the kinetic method occurs at the ortho but not the para position to the phenolic group. However, in the titrimetric procedure, complete electrophilic substitution occurs when bromate with excess bromide is added to the aqueous acid solution of drug. The unreacted bromate-bromide is determined by adding excess iodide and titrating the released iodine with standard thiosulfate solution.

For authentic drug, 1 mL 0.1N bromine solution equals 3.3901 mg phenylephrine hydrochloride, 2.2395 mg salicylamide, or 5.0316 mg tetracycline hydrochloride. (The compendial figure for phenylephrine hydrochloride is 3.3945. Applying statistical analysis, the 2 figures are comparable and no significant difference exists; *t*(calc) equals 0.3106, where *t* = difference between the 2 values/(SD/$\sqrt{\text{no. of exp.}}$) and does not exceed *t* theoretical (t_0 0.975 is 2.571). Therefore the null hypothesis is acceptable.) The standard deviations for 6 replicate determinations were 0.0347, 0.0100, and 0.0285 for phenylephrine hydrochloride, salicylamide, and tetracycline hydrochloride, respectively.

Applying the equation, 1 mL 0.1N bromine solution = molecular weight of drug/(Z × 10 × 1000) g drug, or molecular weight of drug/(Z × 10) mg drug, where Z is the number of bromine atoms involved in the electrophilic substitution reaction, we found that 6 bromine atoms are necessary for bromination of phenylephrine hydrochloride or salicylamide, and 10 bromine atoms for tetracycline hydrochloride.

Interference from lactose, starch, talc, and magnesium stearate, substances that are apt to be present together in capsules or tablets, was investigated by both methods. None of these auxiliary substances exhibited interference when added in an amount equivalent to 0.2 of the concentration of the drug. Chlorobutanol, a preservative added in the same concentration as phenylephrine hydrochloride in nose drops, did not interfere in either method. Although lactose and chlorobutanol are reducing agents, they behaved passively during the reaction with the oxidant bromine under the experimental conditions described here. This may be because the bromine generated in the presence of acid is a weak electron acceptor and therefore is not powerful enough to oxidize either lactose or chlorobutanol. The small amount of ethanol used to dissolve the salicylamide did not interfere in either method.

To assess the accuracy of the methods, a known amount of phenylephrine hydrochloride was combined with an equal amount of chlorobutanol, and known amounts of salicylamide or tetracycline hydrochloride were combined with 0.2 of their concentration of tablet or capsule base ingredients (lactose, starch, magnesium stearate, and talc). Analytical results on these preparations are presented in Table 1. The value for *t*(calc) does not exceed *t*(theor), so the percent recovery is in agreement with the amount added and both methods are accurate. For assays of

Table 2. Assay of commercial dosage forms by kinetic and titrimetric methods

Sample	Kinetic method			Titrimetric method	
	Taken, g(range)	Found, mean % label ± SD (*n*)	*F* value	Taken, g(range)	Found, mean % label ± SD (*n*)
Phenylephrine nose drops[a]	0.010–0.020	105.22 ± 0.58 (6)	0.33[b]	0.015–0.025	104.88 ± 0.96 (5)
Salicylamide tablets[c]	0.080–0.160	101.89 ± 0.96 (8)	1.88[d]	0.020–0.040	101.96 ± 0.70 (8)
Tetracycline capsules[e]	0.020–0.100	99.71 ± 1.36 (15)	2.43[d]	0.030–0.050	99.75 ± 0.87 (12)

[a] Fenox nose drops from Boots Co. Ltd, UK, Lot L3, label claim 0.5% phenylephrine hydrochloride and 0.5% chlorobutanol.

[b] Calculated *F* value, *F* theoretical at 5% level is 5.19.

[c] Cidal Forte tablets from CID Co., Egypt, Lot 1575, label claim 0.5 g salicylamide/tablet.

[d] Calculated *F* value for which *F* theoretical at 5% level is 2.56.

[e] Tetracycline capsules from Pharmchim, Bulgaria, Lot 53, label claim 0.25 g tetracycline hydrochloride/capsule.

commercial samples (Table 2), the results for nominal contents are within the pharmacopeial limits (32, 36). As indicated by the *F*-test (Tables 1 and 2), the methods are of equal precision.

Both methods are accurate and reproducible and do not require expensive instrumentation. Therefore, they are well suited for routine analysis and quality control of phenylephrine hydrochloride in nose drops, salicylamide in tablets, and tetracycline hydrochloride in capsules.

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High Performance Liquid Chromatographic Determination of Clotrimazole in Pharmaceutical Formulations

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A simple stability-indicating high performance liquid chromatographic (HPLC) method has been developed which separates clotrimazole from impurities and decomposition products in bulk drugs, creams, tablets, and solutions. Average recovery data for drug substance added to placebos were: tablet, 99.8%; solution, 99.5%; and cream, 100.0%. Average reproducibilities (RSD) on drug substance and formulations were: drug substance, 1.3%; tablets, 1.8%; solutions, 1.1%; and creams, 0.6%. HPLC assay results for both fresh and degraded samples agree with USP XX titration assay results. The method allows for the simultaneous determination of (*o*-chlorophenyl)-diphenylmethanol hydrolysis product impurity.

Clotrimazole, a synthetic antimycotic agent first described in 1969 (1-3), is currently available in tablet, cream, and solution formulations. The USP XX method (4) for determining clotrimazole in these dosage forms specifies a complexation titration with sodium lauryl sulfate. The reaction is carried out in a 2-phase (acidic aqueous-chloroform) system, and the end point is determined in the chloroform phase with methyl yellow as the indicator. Although the method is simple and stability indicating, it does not lend itself readily to automation. Also, additional methodology is required to determine impurity levels, specifically (*o*-chlorophenyl)diphenylmethanol.

This paper describes a high performance liquid chromatographic (HPLC) method for the determination of clotrimazole and its major degradation product in pharmaceutical dosage forms and drug substance.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Waters Associates M-6000A pump (Waters Associates, Inc., Milford, MA 01757) used at 1.0 mL/min flow rate, Waters Model 710A automatic sampler programmed to inject 20 μL, MPLC RP-18 guard column 3 cm × 4.6 mm id (Brownlee Labs, Inc., Santa Clara, CA 95050), μBondapak C_{18} column (30 cm × 3.9 mm id, Waters Associates), Waters Model 440 fixed wavelength detector operated at 254 nm and sensitivity of 0.2 absorbance unit full scale (AUFS), and Linear Model 485 recorder (Linear Instruments Corp., Irvine CA, 92664) at an input span of 10 mV. Data acquisition and peak processing were performed by a PDP 11/34 Minicomputer utilizing Peak-11 software (Digital Equipment Corp., Maynard, MA 01754).

(b) *Mobile phase.*—Mix 1 part of aqueous 0.025M potassium hydrogen phosphate, dibasic, with 3 parts of HPLC-grade methanol.

(c) *Internal standard solutions.*—(*1*) About 0.7 mg testosterone propionate/mL mobile phase. (*2*) About 0.7 mg testosterone propionate/mL absolute ethanol. (*3*) Dilute internal standard solution 2 exactly 1:10 with absolute ethanol (0.07 mg/mL).

(d) *Reference standard solutions.*—(*1*) Accurately weigh ca 50 mg clotrimazole (USP Reference Standard), add 5.00 mL internal standard solution 1, and dilute to 50.00 mL with mobile phase. (*2*) Accurately weigh ca 50 mg clotrimazole into 100 mL volumetric flask, add 5.00 mL internal standard solution 2, and dilute to volume with absolute ethanol. Mix well.

(e) *Lotrimin® and Gyne-Lotrimin® formulations.*—Schering-Plough Corp.

Preparation of Samples

(a) *Bulk drug substance.*—Prepare sample in manner described for reference standard solution 1. Continue as directed under *Procedure*.

(b) *Solution.*—Use to-contain pipet to transfer sample equivalent to 50 mg clotrimazole into 50 mL volumetric flask and use mobile phase to rinse pipet into flask. Add 5.00 mL internal standard solution 1, dilute to volume with mobile phase, and mix well. Continue as directed under *Procedure*.

(c) *Tablets.*—Accurately weigh 10 tablets, determine average unit weight, and grind to fine powder. Accurately weigh an amount of powder equivalent to 100 mg clotrimazole, and transfer to 50 mL centrifuge tube. Add 10.00 mL internal standard solution 1. Extract sample twice as follows: Add ca 25 mL mobile phase to tube, rotate tube for 15 min, centrifuge, and

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transfer supernate to 100 mL volumetric flask. Then dilute approximately to volume with mobile phase, and mix well. Continue as directed under *Procedure*.

(d) *Cream.*—Accurately weigh thoroughly mixed cream equivalent to 10 mg clotrimazole into 50 mL centrifuge tube and add 10.00 mL internal standard solution 3. Heat 5 min in 50°C water bath with occasional shaking. Remove from bath and shake vigorously until cooled to room temperature, then place in an ice–methanol bath 15 min. Centrifuge 5 min and transfer supernate to 25 mL stoppered container. Repeat extraction with ca 10 mL absolute ethanol. Continue as directed under *Procedure*.

Procedure

Chromatograph 20 μL aliquots of sample and reference standard preparations by using chromatographic parameters described earlier. For bulk drug substance, solution, and tablets assay against reference standard solution 1. Assay cream samples against reference standard solution 2. Approximate retention times are 9 and 14 min for clotrimazole and testosterone propionate, respectively.

Calculations

Calculate clotrimazole as follows:

$$\text{Drug substance (\%)} = (R_{sam}/R_{std}) \times (W_{std}/W_{sam}) \times 100$$

$$\text{Solution (mg/mL)} = (R_{sam}/R_{std}) \times (W_{std}/V_{sam})$$

$$\text{Tablet (mg/tablet)} = (R_{sam}/R_{std}) \times (W_{std}/W_{sam}) \times AUW$$

$$\text{Cream (mg/g)} = (R_{sam}/R_{std}) \times (W_{std}/W_{sam}) \times D$$

where R_{sam} = ratio of clotrimazole peak height to testosterone propionate peak height in sample chromatogram, R_{std} = peak height ratio in standard chromatogram, W_{std} = weight of clotrimazole in the reference standard solution, W_{sam} = weight of sample, V_{sam} = volume of sample, AUW = tablet average unit weight, D = dilution factor (D = 0.2 for cream assay).

Results and Discussion

The criteria for developing a successful HPLC determination of clotrimazole in pharmaceutical dosage forms were as follows: The method should be stability indicating, free of interference from excipients, applicable to a wide variety of dosage forms, and robust and straightforward enough for routine use in quality control laboratories.

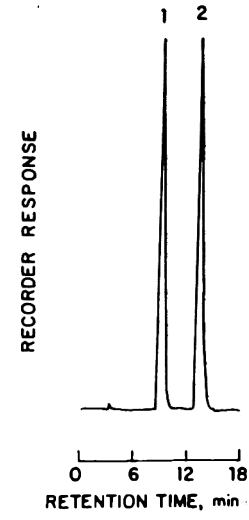
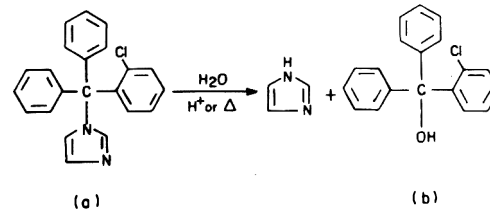


Figure 1. High performance liquid chromatogram of clotrimazole extracted from clotrimazole tablets: 1, clotrimazole; 2, internal standard.

No interference from formulation excipients was observed. Figures 1–3 show representative chromatograms of clotrimazole tablet, solution, and cream samples. At the analysis wavelength of 254 nm, the only absorbing tablet excipient is povidone; this compound is not appreciably soluble in the extracting solvent. The solution has no UV-absorbing excipients, but the cream formulation contains benzyl alcohol; this preservative has a retention time of about 2 min and does not interfere with the clotrimazole or internal standard peak.

Possible interferences from clotrimazole degradation products are separated by the proposed HPLC method. Büchel et al. (5) proposed the degradation pathway shown in Scheme 1, where the primary degradation product of clo-



Scheme 1. Degradation pathway of clotrimazole: (a) clotrimazole, (b) (*o*-chlorophenyl)diphenylmethanol.

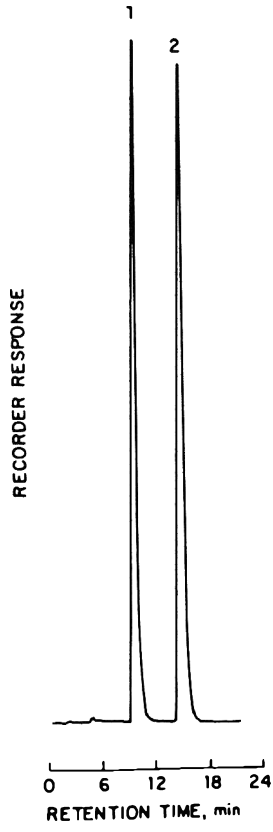


Figure 2. High performance liquid chromatogram of clotrimazole obtained from clotrimazole solution: 1, clotrimazole; 2, internal standard.

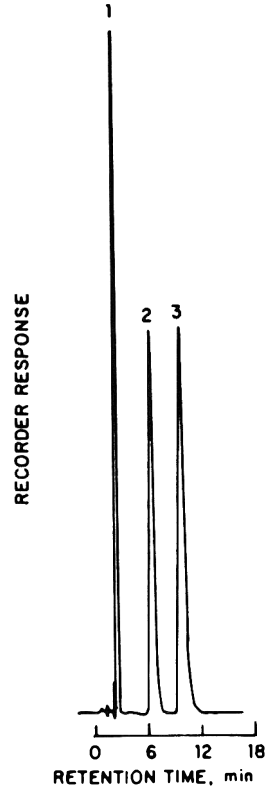


Figure 3. High performance liquid chromatogram of clotrimazole extracted from clotrimazole cream: 1, benzyl alcohol (preservative); 2, clotrimazole; 3, internal standard.

trimazole (a) results from hydrolysis of the imidazole group to form (*o*-chlorophenyl)diphenylmethanol (b). The imidazole fraction, which has a retention time of about 2 min in this system, has virtually no UV absorptivity at 254 nm and does not appear in the chromatograms. The degradation product (b) chromatographs faster than, and is separated from, clotrimazole. Typical chromatograms of samples subjected to accelerated degradation are shown in Figures 4–6. Under these extreme temperature conditions, several minor clotrimazole decomposition products are formed in addition to (*o*-chlorophenyl)diphenylmethanol; all degradation products are well separated from clotrimazole and the internal standard.

Response of the chromatographic system tested over a wide concentration range shows excellent linearity. Peak height ratios of clotrimazole to internal standard were measured at constant concentration of internal standard and clotrimazole concentrations ranging from 0.1 to 2.0 mg/mL. For clotrimazole concentrations (mg/mL) of 0.101, 0.252, 0.503, 1.006, 1.509, and 2.013, relative detector response was 0.00615, 0.0156, 0.0311, 0.0624, 0.0939, and 0.1256, re-

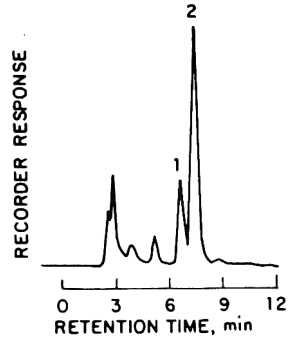


Figure 4. High performance liquid chromatogram of clotrimazole and clotrimazole decomposition products extracted from clotrimazole tablets stored for 2 weeks at 95°C: 1, decomposition product [(*o*-chlorophenyl)diphenylmethanol]; 2, clotrimazole. Other decomposition peaks unidentified.

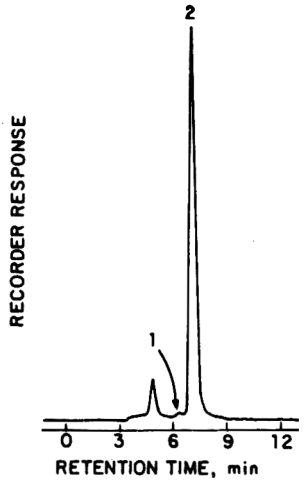


Figure 5. High performance liquid chromatogram of clotrimazole and clotrimazole decomposition products obtained from clotrimazole solution stored for 2 weeks at 75°C: 1, decomposition product [(*o*-chlorophenyl)diphenylmethanol]; 2, clotrimazole. Other decomposition peaks unidentified.

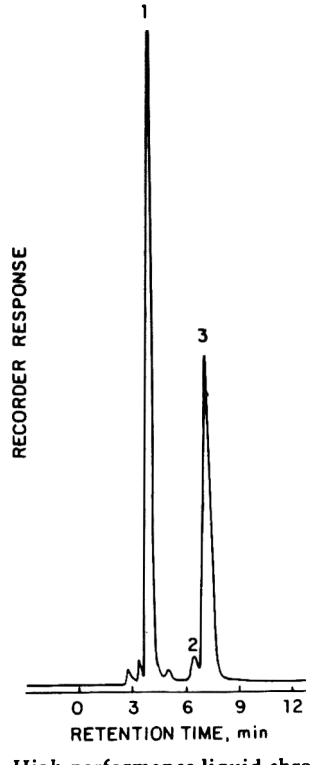


Figure 6. High performance liquid chromatogram of clotrimazole and clotrimazole decomposition products obtained from clotrimazole cream heated for 10 days at 75°C: 1, benzyl alcohol (preservative); 2, decomposition product [(*o*-chlorophenyl)diphenylmethanol]; 3, clotrimazole.

Table 1. Effect of sample size on clotrimazole determination in dosage forms

Formulation	Sample size	Found, mg	% Labeled amt
Tablet	0.5179 g	50.4	97.8
	1.0641	104.1	98.3
	1.5135	149.9	99.5
	1.9993	196.9	98.9
Tablet[a]	0.5046 g	34.8	68.5
	1.0000	68.1	69.1
	1.4953	103.4	68.8
	2.0003	136.3	67.8
Solution	2.00 mL	20.55	102.8
	3.00	30.75	102.5
	5.00	51.00	102.0
	7.00	70.55	100.8
	10.00	101.4	101.4
Solution[b]	2.00 mL	18.35	91.8
	3.00	28.35	94.5
	5.00	45.95	91.9
	7.00	64.05	91.5
	10.00	91.80	91.8
Cream	0.5183 g	5.18	99.9
	1.0279	10.40	101.2
	1.5100	15.48	102.5
	2.0332	20.56	101.1
Cream[c]	0.5238 g	5.01	95.6
	1.0215	9.69	94.9
	1.5602	14.97	95.9
	2.0319	19.34	95.2

[a] Stored for 2 weeks at 95°C.
[b] Stored for 2 weeks at 75°C.
[c] Stored for 10 days at 75°C.

spectively. Response was rectilinear (R >0.9999); there is no reason to believe that there is a bias.

The proposed sample preparation procedures are designed to be insensitive to the size of sample taken. The data in Table 1 demonstrate that, over the range studied, assay results are independent of sample size. For the procedural amount of tablet sample, a single extraction with mobile phase gave recoveries >98%; the second extraction was added to ensure quantitative extraction. In preparation of cream samples, 2 ethanol extractions were necessary to quantitatively remove the internal standard from the sample matrix. All samples prepared according to the procedures are stable for at least 24 h; this stability is essential for purposes of automated analysis.

In the analyses of cream samples, accumulation of excipients on the analytical column causes a marked decrease in efficiency. For this reason an RP-18 precolumn is used to protect the analytical column. By using such a precolumn, this laboratory could make more than 100 injections of cream samples with no measurable decrease in column efficiency.

Table 2. Recovery of clotrimazole added to formulation placebos

Formulation	Added, mg	Found, mg	Rec., %
Tablet	51 8	52.3	101.1
(1.0 g placebo)	101.2	101.4	100.2
	153.2	151.1	98.7
	202 9	200.9	99.0
Tablet	51 0	50.9	99.7
(1.0 g placebo)[a]	101 6	101.6	100.0
	156 5	155.0	99.1
	200 0	202.4	101.2
Solution	20 05	19.94	99.4
(5.00 mL placebo)	30.05	29.60	98.5
	50 10	49.75	99.3
	75 15	75.10	99.9
	100 2	100.4	100.2
Solution	25 05	24.58	98.1
(5.00 mL placebo)[b]	50 05	49 45	98.8
	75 10	74.85	99.7
	100.1	100.3	100.1
Cream	4 76	4.75	99.8
(1.0 g placebo)	9 51	9.49	99.8
	14 27	14.30	100.2
	19 02	19.10	100 4
Cream	5.04	5.00	99.2
(1.0 g placebo)[c]	10 08	10.15	100.7
	15 12	15.22	100.7
	20 16	20.37	101.0

[a] Placebo stored for 2 weeks at 95°C.
[b] Placebo stored for 2 weeks at 75°C.
[c] Placebo stored for 10 days at 75°C.

Table 3. Reproducibility of clotrimazole determinations in bulk drug and dosage forms

Day	Found, % labeled amt		
	Bulk Drug		
	Lot A	Lot B	Lot C
1	102.2	98.8	101.2
2	101.0	101.2	101.2
3	99.4	99.6	99.0
Av.	100.9	99.9	100.5
RSD, %	1.4	1.2	1.3
	Tablet		
	Lot D	Lot E	Lot F[a]
1	97 8	98.6	68.5
2	100.6	100.0	72.1
3	98.0	98.7	68.8
4		96.7	69.4
Av.	98.8	98.5	69.7
RSD, %	1.6	1.4	2.4
	Solution		
	Lot G	Lot H	Lot I
1	101.9	101.8	100.6
2	97.6	102.9	101.7
3	99.0	102.8	102.7
4	98.8		
Av.	99.3	102.5	101.7
RSD, %	1.8	0.6	1.0
	Cream		
	Lot J	Lot K	Lot L
1	100.9	101.8	102.2
2	101.1	102.1	102.7
3	102.2	103.9	102.3
4	102.1	102.2	
Av.	101.6	102.5	102.4
RSD, %	0.7	0.9	0.3

[a] Sample stored for 2 weeks at 95°C.

Recovery studies were performed by adding increasing amounts of clotrimazole to the appropriate placebos and proceeding with sample preparation as described earlier. Both normal and heat-treated placebos were used to test for assay bias. As the results in Table 2 indicate, recoveries range from 98 to 101% and show negligible bias.

Reproducibility of the assay methods was tested by performing replicate assays on several batches of each formulation over a period of several days. Day-to-day reproducibility, as evidenced by Table 3, is excellent, with relative standard deviations ranging from 0.3 to 1.8%, except for one lot of thermally degraded tablets for which the relative standard deviation was 2.4%.

Chromatographic and USP XX titrimetric results are compared in Table 4. Compendial assay specifies nonaqueous titration with perchloric acid for the bulk drug substance and titration with sodium lauryl sulfate for clotrimazole in dosage forms. Agreement between the present and compendial methods is excellent for both bulk drug and dosage form assays.

The HPLC method provides a means for determining not only clotrimazole levels, but (*o*-chlorophenyl)diphenylmethanol levels as well. These simultaneous measurements eliminate the need for additional laboratory work as required in the compendial thin layer chromatographic estimation of the hydrolysis product. The frac-

Table 4. Comparison of determination of clotrimazole in bulk drug and dosage forms by HPLC and compendial assays

Formulation	Lot	Assay	
		USP XX	HPLC
Bulk drug	A	100.5 %	100.9 %
	B	100.5	99.9
	C	100.2	100.5
Tablet	D	98.0 mg/tablet	98.8 mg/tablet
	E	97.4	98.5
	F[a]	70.2	69.7
Solution	G	10.03 mg/mL	9.93 mg/mL
	H	10.28	10.25
	I	10.29	10.17
Cream	J	10.19 mg/g	10.16 mg/g
	K	10.02	10.25

[a] Sample stored for 2 weeks at 95°C.

tion of degradation product (b) is calculated as the peak height ratio of (*o*-chlorophenyl)diphenylmethanol to clotrimazole. This approximation is based on the similar absorptivities at 254 nm (clotrimazole $a \approx 1.73$ mL/mg·cm, (*o*-chlorophenyl)diphenylmethanol $a \approx 1.75$ mL/mg·cm) and retention times of the 2 compounds. The estimation can be used reliably for (*o*-chlorophenyl)diphenylmethanol levels of 0.5–10% relative to the clotrimazole content.

Conclusion

The liquid chromatographic method described in this paper was used to determine clotrimazole in the bulk drug material and in various dosage forms. This method provides a simple, accurate, and precise assay for clotrimazole and allows estimation of the hydrolysis product (*o*-chlorophenyl)diphenylmethanol.

Acknowledgments

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High Performance Liquid Chromatographic Analysis of Hydrocortisone Acetate Ointments: Interlaboratory Study

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An interlaboratory study was carried out on a high performance liquid chromatographic method for determining hydrocortisone acetate in ointments. The method represents an alternative to the colorimetric procedure of the *British Pharmacopoeia*. Two samples of a commercially available hydrocortisone acetate ointment were analyzed by 14 laboratories. Column performance and precision of the assay were satisfactory. The total error standard deviation for the method was 3.69%.

The present Australian official method for determining hydrocortisone acetate in ointments is that of the *British Pharmacopoeia* (BP) which specifies a colorimetric (tetrazolium) assay (1). This method is time-consuming and subject to interference from a number of sources. To overcome these shortcomings, a high performance liquid chromatographic (HPLC) method was developed (2). The HPLC method uses rapid extraction of the ointment with chloroform followed by liquid–solid chromatography on a silica gel column, using an isopropanol–cyclohexane mobile phase. Previous work in this laboratory (2) has indicated that the method gives results that are comparable with those obtained by using the BP procedure, but the HPLC method is more precise and more rapid. This communication describes an interlaboratory study of the HPLC method.

Experimental

Dienestrol was selected as an internal standard for the assay on the basis of its retention volume. Because of the quality of the separation obtained in our laboratory, we decided to set a resolution factor, R, lower limit of 1.6 for the separation of the hydrocortisone acetate and dienestrol peaks. Suggested limits of 2% were set for the coefficients of variation of R and of the peak height ratio obtained from replicate injections.

Column dimensions of 25 cm × 2 mm id were suggested. The mobile phase was given as isopropanol–cyclohexane (10 + 90, v/v) but participants were permitted to adjust these proportions to meet the resolution criteria. Typical values for pressure and flow rate were included in the protocol as a guide.

The original work on the method was carried out using a variable wavelength detector at 240 nm. The trial protocol allowed the use of a fixed wavelength detector at 254 nm as an alternative. A minimum height of 60% full scale deflection was required for each peak. Peak height rather than area was specified on the grounds that not all laboratories would have suitable integrators and that peak widths were expected to be relatively narrow. It was expected that only hydrocortisone acetate would be present in the ointments but participants were also requested to report the peak height ratio of any hydrocortisone peak observed.

In this trial a commercially available brand of a 1% hydrocortisone acetate ointment was used because it was considered impracticable to suitably prepare an ointment of known composition. Tubes of the ointment from 2 recent production batches were purchased, and these represented the sample pair. Because of possible homogeneity problems, which had been encountered with other topical preparations during the routine sampling program conducted here, we decided to analyze each sample in duplicate (2 tubes) and to assay portions from the top and the bottom of each tube. In this way, it was hoped that any contribution of sample nonhomogeneity to apparent analytical error would be recognized and overcome. These data might also provide useful information regarding the distribution of active substance within the tubes. The tubes were to be sampled by squeezing out portions of approximately 5 g from the top and bottom of the tube, discarding the "middle" portion of 15–20 g. The portions to be analyzed were stirred (rod or spatula) for 1 min before weighing and dissolution of 0.5 g of the ointment.

Sixteen laboratories, including National Biological Standards Laboratory (NBSL), took part in the study, which was carried out in 3 stages. All laboratories received reference substances from the same source, which were checked for purity by HPLC before distribution. In the first stage, laboratories prepared a calibration solution of hydrocortisone acetate and dienestrol and used this to obtain data on the peak height ratio and the resolution factor. Minimum criteria for

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peak height, resolution, and reproducibility had to be met before proceeding to the second stage of analyzing a preliminary sample. The preliminary samples used in the trial were tubes of ointment previously tested by this laboratory during the official sampling program. This sample was intended to give participants experience with the method, and preliminary assay results were not required to be reported. Laboratories were asked to contact NBSL after the preliminary sample had been successfully tested and the response and resolution criteria had been met. Test samples were then sent to participants who were asked to report the 8 assay results (top and bottom of the 2 tubes from each sample), the resolution factor, coefficient of variation of the peak height ratio of the calibration solution and peak height ratio for hydrocortisone:dienestrol if hydrocortisone was detected. Laboratories were asked to contact NBSL if any difficulties were encountered or if any modifications to the method were desired.

Results

Measurement of Column Performance

Two of the laboratories were unable to meet the criteria for resolution and/or reproducibility and did not proceed to analysis of the test samples. In these cases, replacement of the column seemed appropriate, but was not feasible for the organizations concerned.

In analyzing the test samples, participants easily met the conditions for resolution factor although several laboratories had to use solvent proportions different from those specified. R values reported ranged from 2.55 to 5.77 with a mean of 3.87. More difficulty was experienced in achieving a reproducible peak height ratio. The minimum figure of 2% was specified on the basis of previous work at NBSL, and also from consideration of typical values used in USP monographs. Five laboratories reported results for coefficient of variation in excess of 2% with Laboratory 2 obtaining results in excess of 3%. The lowest coefficient of variation obtained was 0.39% (Laboratory 12).

Evaluation of the Method

Individual assay results are shown in Table 1. Using the procedure described by Steiner (3), the experimental variation between laboratories and between replicates was shown to be homogeneous. Because there is no way of knowing the actual content of active substance in these samples, and because it has not been possible to check the interlaboratory mean values by another method, these mean values have been regarded for the purposes of this study as representing the true mean contents of hydrocortisone acetate in the samples.

The results given in Table 1 differ from those reported in 2 instances. Laboratory 4 reported the presence of significant quantities of hydrocortisone in the samples. The results in Table 1 have therefore been calculated on the basis of both hydrocortisone acetate and hydrocortisone to give a "total hydrocortisone" value, using the peak height data provided for each compound. Decomposition of hydrocortisone acetate in the ointment was not noted by any other laboratory, and it is possible that in this instance the samples were subjected to heat stress during storage or were sensitive to some impurity in the solvents used in the mobile phase or during sample preparation.

The values for Sample B, Tube 1, bottom, and Tube 2, top, from Laboratory 5 are each the first of duplicate determinations made on these portions. The second determinations were carried out as a check on the apparently high results and gave values of 108.0 and 109.8%, respectively.

Rank orders for the laboratories in terms of the mean of the results for each tube are shown in Table 2. Laboratory 14 shows a consistently high ranking and Laboratory 2 is consistently low. Results from Laboratory 14 were rejected on the basis of Youden's test for outliers (3). From the remaining results, mean values for content of active substance as percent of stated content are 98.3% for Sample A and 99.4% for Sample B. Youden (3) represents the precision (or repeatability) standard deviation by S_R, the bias (reproducibility) standard deviation by S_B, and the total error standard deviation by S_D. The values for S_R, S_B, and S_D for the method were 1.45, 2.40, and 3.69, respectively.

Within-Tube Variation — Sample Homogeneity

The sampling plan used was adopted, in part, to minimize possible errors due to nonhomogeneity. As it turned out, nonhomogeneity did not appear to present a problem with the ointment samples used. When all results are considered, use of a paired t-test indicates that there was no significant difference between results for tops and bottoms of individual tubes or between tops and bottoms of different tubes for either sample ($P < 0.15$). Both samples appear to be homogeneous and, in fact, there is no significant difference between Sample A and Sample B (t-

Table 1. Results (% stated content) for interlaboratory study of HPLC analysis of hydrocortisone acetate ointment

Lab.[a]	Tube 1 Top	Tube 1 Bottom	Tube 2 Top	Tube 2 Bottom	Mean	Std dev.[b]
			Sample A			
1	—[c]	100.0	98.0	103.0	100.33	
2	92.6	98.3	94.1	89.4	93.60	
3	101.27	101.0	97.8	102.6	100.68	
4[d]	105.39	92.2	98.1	96.7	98.10	
5	100.9	98.2	100.9	98.8	100.20	
6	100.3	99.0	102.0	102.5	100.95	
7	97.6	92.1	97.6	93.5	95.20	
8	94.6	94.6	97.5	94.4	95.28	
9	98.68	96.5	91.0	95.7	95.23	
10	99.1	98.3	101.3	99.3	99.50	
11	100.81	101.1	96.9	98.9	99.40	
12	88.3	98.6	99.6	99.1	98.68	
13	99.14	98.7	100.3	100.3	99.60	
14	106.3	108.2	108.1	106.0	107.15	
			Sample B			
1	107.0	104.0	109.0	106.0	106.50	3.89
2	92.4	96.9	91.6	93.3	93.55	2.86
3	101.5	98.6	99.5	100.8	100.09	1.61
4[d]	94.9	94.4	98.4	102.6	97.59	4.37
5	99.70	105.0[e]	106.0[e]	96.8	101.88	3.22
6	100.0	99.5	101.4	100.2	100.28	1.23
7	95.7	97.9	100.0	98.3	97.98	2.64
8	94.6	95.8	97.1	97.4	96.23	1.38
9	91.8	108.7	101.3	94.0	98.93	5.75
10	97.7	99.0	100.2	99.7	99.40	1.11
11	101.5	100.9	101.6	101.4	101.35	1.63
12	100.0	100.7	99.7	99.8	100.05	4.06
13	98.2	99.3	100.1	98.2	98.95	0.88
14[f]	113.2	113.2	114.9	104.5	111.45	3.92

Overall mean for Sample A	98.3
Overall mean for Sample B	99.4
S_D	3.69
S_R	1.45
S_B	2.40

[a] The order of participating laboratories in Tables 1–3 does not correspond to the order shown in *Acknowledgments*.
[b] Standard deviations refer to all results for each laboratory.
[c] Lost, handling error.
[d] Results calculated on the total hydrocortisone content, using both hydrocortisone acetate and hydrocortisone peaks.
[e] Results are the first of duplicate determinations made on these subsamples.
[f] Results rejected on the basis of Youden's test for outliers (3) not included in overall mean for Sample B.

test) ($P < 0.4$). This overall assessment does not preclude the possibility that the contents of individual tubes were not homogeneous. For example, the results for Sample B reported by Laboratory 5 suggest that this may have occurred in some cases. There is a possibility that nonhomogeneity could develop during storage of some topicals, which poses potential problems both for the manufacturing industry and for controlling authorities.

Because, overall, the samples can be considered to be homogeneous and identical with respect to content of active substance, the 8 results reported by each laboratory can be regarded as replicates and the variance for each set of 8 results gives a further and more detailed indication of the precision achieved by each operator. There may be a nonhomogeneity component to this indicator but this is likely to be small. Coefficients of variation for each set of results are given in Table 3. Laboratories with a high coefficient of variation for sample results tend to also have a high coefficient of variation for the peak height ratios obtained from replicate injections. This gives some indication of the contribution of instability of column conditions to precision errors.

Table 2. Rank order for laboratories, based on the means of results for top and bottom samples from each tube

	Sample A		Sample B		
Lab.	Tube 1	Tube 2	Tube 1	Tube 2	Total
1	4	4	2	2	12
2	12	14	13	14	53
3	2	7	7	6	22
4	8	10	13	6	37
5	6	3	3	4	16
6	5	2	8	5	20
7	13	12	11	10	46
8	14	11	12	13	50
9	11	13	6	12	42
10	9	5	9	8	31
11	3	8	4	3	18
12	10	8	5	9	32
13	7	5	10	10	32
14	1	1	1	1	4

Comparison with the Official Method

In addition to the HPLC assays, Samples A and B were also analyzed at NBSL by the BP method (1). Eight replicate determinations were carried out on each sample, with all color developments, including blanks and standards, performed in triplicate. Mean results by this method were 101.9% of stated content, with a standard deviation (SD) of 5.6, and 98.8%, SD 7.5, for Samples A and B, respectively. These results were similar to those obtained at NBSL (Lab. 3, Table 1) for the HPLC method (100.7%, SD 2.0, and 100.1%, SD 1.3, for Samples A and B, respectively), confirming previous comparative work on these methods (2). The precision of the BP procedure was appreciably poorer than that of the HPLC method.

Table 3. Coefficients of variation (CV) for all sample results and for peak height ratio of the calibration solution

Lab.	Sample results, CV%	Peak height ratio, CV%
1	3.8	2.4
2	3.1	3.1
3	1.6	1.5
4	4.5	2.9
5	3.3	0.7
6	1.2	0.4
7	2.7	1.2
8	1.4	0.7
9	5.9	2.1
10	1.0	0.4
11	1.1	0.7
12	0.8	2.0
13	0.9	2.4
14	3.6	1.4

Comments Received from Participating Laboratories

The most important points raised concern the instability and solubility of the internal standard and the proportions of solvent in the mobile phase. Some laboratories had difficulty in dissolving the internal standard and found that heating or ultrasonication was necessary. Four laboratories found that dienestrol decomposed to some extent in the solutions being used, necessitating the use of fresh solutions. It is apparent that a different internal standard would be desirable, especially in view of the tendency to photodecomposition shown by dienestrol. It should be noted, however, that despite the shortcomings of the internal standard, acceptable results were achieved with the method.

The composition of the mobile phase varied significantly between laboratories. Laboratory 11 commented that the concentration of isopropanol could be a very important factor, which might vary from machine to machine and column to column. This seems very pertinent and when the method is circulated for wider use, a range of solvent proportions will be specified with minimum column performance criteria. It is accepted that laboratories must be free to adjust mobile phase composition to achieve satisfactory resolution, but it would seem necessary to set limits to this adjustment and avoid effectively different methods being used in a referee situation. From work done in other interlaboratory studies now in progress, it seems that a question of varying solvent proportions will also be of great relevance to reverse phase separations and will be related to brands of column packings and new column technologies.

One laboratory suggested that if hydrocortisone was present in a sample, the analysis time would be as long as 30–40 min. We believe that the analysis time would in fact be shorter than this and acceptable for an official test method, as opposed to a quality control procedure. The official method will specify an extended running time in order to demonstrate the absence of hydrocortisone.

Three laboratories reported slight cloudiness, with the samples in the sample solutions tending to precipitate. This was easily remedied by warming the solutions. Three laboratories had comments concerning the weighing and volumetric procedures specified in the protocol.

Five laboratories reported no problems with the method.

Peak heights rather than peak areas were

specified in the method because not all laboratories had suitable integrators. Consideration will be given to making provision for peak heights and peak areas as alternatives. However, whichever method is used, adequate precision should first be demonstrated by means of a calibration sample. The performance of an integrator may cause problems. In this trial, it was noted that the results from Laboratory 11 were higher when calculated on an area basis, but that the reported integrator-based peak area ratios did not agree very closely with the ratios based on manual area measurement. On the basis of the results from all laboratories, a limit of 2.4% for the coefficient of variation of peak height ratio seems appropriate.

Conclusion

The dosage form tested in this trial presented obvious sampling problems, but potential difficulties from nonhomogeneity did not arise. It is considered that, even with the use of a somewhat unsatisfactory internal standard, the method is acceptable for official testing purposes. We intend to adopt the method for the quantitative analysis of hydrocortisone acetate ointments in our laboratory. Infrared spectrophotometry will be used as the primary method of identification.

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Separation and Characterization of Standard Propoxyphene Diastereomers and Their Determination in Pharmaceutical and Illicit Preparations

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Due to the existence of 2 asymmetric carbon atoms in the propoxyphene molecule, there are 4 diastereomers: alpha dextro, alpha levo, beta dextro, and beta levo. Only α-*d*-propoxyphene is included under the federal Controlled Substances Act. Baseline separations of propoxyphene from various incipients (aspirin, caffeine, phenacetin, and acetaminophen) present in pharmaceutical and illicit preparations, and between the alpha and beta diastereomers, were achieved by high pressure liquid chromatography. The column eluant was collected and propoxyphene was extracted. The optical isomers were differentiated and characterized by melting points and by chemical microcrystalline tests. Using hot stage thermomicroscopy, the eutectic melting points of binary isomeric mixtures of propoxyphene bases and salts were found to be depressed about 10° and 15-30°C, respectively, below the individual isomer melting points. The characteristic microcrystals formed with the alpha racemic mixtures by using a glycerin-aqueous gold chloride reagent were not produced by the beta racemic mixtures.

Of the 4 isomers of propoxyphene, only α-*d*-propoxyphene, α-*d*-4-(dimethylamino)-3-methyl-1,2-diphenyl-2-butanol propionate, is included under the federal Controlled Substances Act, and it is this diastereomer that is the widely prescribed drug for relief of moderate pain. The propoxyphene molecule has 2 asymmetric carbon atoms, which leads to the existence of 2 pairs of optical isomers, called alpha (*d* or *l*) and beta (*d* or *l*) (where *d* indicates dextro and *l* indicates levo isomers). The β (*d* or *l*) isomers have no physiological effect (1); the α-*l* isomer has important antitussive properties (2), but only the α-*d*-propoxyphene isomer possesses analgesic properties (3, 4).

α-*d*-Propoxyphene is manufactured (Eli Lilly and Co. is the major manufacturer) in a variety of dosage forms, both with and without other analgesic components. This laboratory has received many submissions of this drug in the form of commercial tablets, capsules, or simply white powders.

After separation and detection of propoxyphene in these commercial and illicit preparations, one of the challenges facing the forensic chemist is to positively determine if the one federally controlled isomer of the 4 possible isomers is present. The unequivocal answer to this question has not been presented in the literature, although several methods, such as thin layer chromatography (5), spectrophotometry (6, 7), gas chromatography (GC) (8–10) and GC–mass spectrometry (MS) (11), or a combination of these techniques, have been reported for the qualitative and quantitative determination of propoxyphene in biological samples. All of these methods have the disadvantages of being either time-consuming, cumbersome, or both, and lack specificity for detecting α-*d*-propoxyphene. Above all, no attempt was made in these studies to differentiate and characterize the propoxyphene diastereomers. Recently one study has appeared (12) concerning techniques for determining α-*d*-propoxyphene. The present investigation was undertaken to systematically characterize and differentiate propoxyphene isomers in seized pharmaceutical preparations and illicit powders of small quantity without prior extraction and purification, a procedure that has been practiced in our laboratory for 2 years.

We have used reverse phase high pressure liquid chromatography (HPLC) to identify and separate α- and β-propoxyphene from each other and from incipients present in the sample. The eluant from the column, after extraction, may be used to identify propoxyphene (e.g., by GC-MS) and/or further characterize the specific optical isomer present by microchemical tests or by mixed melting point determinations. To differentiate the dextro and levo isomers, the microcrystalline tests of Clarke (13, 14) and Fulton (15) for α-*dl*-propoxyphene were applied to the eluant or the raw sample mixed with standard isomers. Alternatively, the eluant or a sample extract was crystallized, and mixed melting points with standard optical isomers were measured by thermomicroscopy.

METHOD

Apparatus

(a) *High pressure liquid chromatograph.*—Waters Associates (Milford, MA 01757) equipped with

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Model 660 solvent programmer, Model 6000A delivery system, Model U6K injectors, Model 440 fixed wavelength (254 nm) detector connected in series with Model 450 variable wavelength detector operated at 257 nm and used in conjunction with 10 mV recorder (Houston Instrument, Austin TX 78753). Flowcells each with fluid volume of 8 μL.

(b) *HPLC prepacked columns.*—150 mm × 4.6 mm id, 5 μm particle size, Supelcosil LC-18 (Supelco, Inc., Bellefonte, PA 16823), or μBondapak C_{18} (Waters Associates), 300 mm × 3.9 mm id, 10 μm particle size.

(c) *Polarizing microscope.*—Leitz Labolux Pol-D equipped with 10×, 25×, and 40× objectives, N.A.0.90 condenser, combination binocular observation-photography tube, Leica MDa 35mm camera, and Kodak ASA 32 Panatomic-X film (developed in Kodak Polydol at 70°F).

(d) *Hot stage.*—Mettler FP-2. Temperature range 20-300°C; heating rates 10, 2, and 0.2°C/min.

Reagents

(a) *HPLC solvents.*—Methanol (HPLC grade, Fisher Scientific Co.), acetonitrile (HPLC grade, Waters Associates), distilled water. Filter all solvents through 0.45 μm Millipore filters.

(b) *HPLC buffers.*—0.01M tetrabutylammonium hydroxide (Eastman Kodak Co.) adjusted to pH 7 with 85% phosphoric acid, or 0.01M dibasic ammonium phosphate (Baker Analyzed Reagent) adjusted to pH 7 with 85% phosphoric acid, for use with μBondapak C_{18} column, or in combination with 0.01% *N,N*-dimethyloctadecylamine (Eastman Kodak Co.), for use with Supelcosil LC-18 column, all filtered through 0.45 μm Millipore filters.

(c) *HPLC mobile phase.*—Methanol or acetonitrile combined with HPLC buffers as specified in figure legends. Solvents and buffers were mixed automatically in reference manifold of Model 6000A pump according to solvent programmer conditions.

(d) *Sample standards.*—Supplied by Eli Lilly and Co. (Indianapolis, IN 46206): α-*d*-propoxyphene.HCl, α-*l*-propoxyphene napsylate (2-naphthalene sulfonate), β-*dl*-propoxyphene.HCl. Purchased from local suppliers: Darvon®, H03 (Lilly), 65 mg α-*d*-propoxyphene.HCl, USP. Darvon with A.S.A.®, H04 (Lilly), 65 mg α-*d*-propoxyphene.HCl, USP and 325 mg aspirin. Wygesic®, 85 (Wyeth Laboratories, Philadelphia, PA 19101), 65 mg α-*d*-propoxyphene.HCl and 650 mg acetaminophen. Darvon Compound-65, H06 (Lilly), 65 mg α-*d*-propoxyphene.HCl, USP with 227 mg aspirin, 162 mg phenacetin, and 32.4 mg caffeine. Darvon-N®, C53 (Lilly), 100 mg α-*d*-propoxyphene napsylate, NF; Darvon-N with A.S.A., C54 (Lilly), 100 mg α-*d*-propoxyphene napsylate, NF and 325 mg aspirin. Darvocet-N® 50, C51 (Lilly), 50 mg α-*d*-propoxyphene napsylate, NF and 325 mg acetaminophen. Novrad®, H14 (Lilly), 100 mg α-*l*-propoxyphene napsylate, NF.

(e) *Gold chloride microcrystal test reagent.*—Dissolve 1 g commercially available gold chloride, $HAuCl_4.3H_2O$, in solution of 2 mL glycerin and 18 mL water.

(f) *Melting point standards.*—Mettler Thermometric Standards: naphthalene (mp 80.25 ± 0.05°C), adipic acid (mp 151.40 ± 0.05°C), 2-chloroanthraquinone (mp 209.00 ± 0.05°C), and anthraquinone (mp 284.50 ± 0.05°C).

(g) *Extraction reagents.*—Hexanes (Fisher Certified reagent), benzene (Fisher Certified ACS), chloroform (Baker Analyzed Reagent), sodium bicarbonate (Baker Analyzed Reagent).

HPLC Determination

Thoroughly shake 35 mg Darvon (H03) powder, 135 mg Darvon Compound-65 (H06) powder, 88 mg crushed tablet of Darvocet-N 50 (C51) (after removing red coating), or 50-100 mg illicit powder with 1 mL solvent. Water was used to advantage as solvent to dissolve hydrochloride salts of propoxyphene present in commercial preparations; methanol was the solvent of choice for napsylate salts. Centrifuge sample solutions and then pass through 0.45 μm Millipore filters before injection onto column.

Inject 3 μL clear sample solution with mobile phase flow rate of 1 mL/min through Supelcosil LC-18 column or with flow rate of 2 mL/min through μBondapak C_{18} column. Use 5 mm/min chart speed. Use sensitivity of 0.1 absorbance unit full scale (AUFS) unless otherwise specified. Operate HPLC columns at ambient temperature.

Column eluant may be collected to recover pure propoxyphene for further characterization by GC-MS, mixed melting points, or microcrystalline tests. Increase injection load 4 to 5 times and air-dry eluant to eliminate organic solvent. Make resulting aqueous solution basic (pH 8) with $NaHCO_3$ and extract twice with an equal volume of hexanes. Evaporate hexanes layer to dryness to obtain crystals as detailed below.

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Alternative Sample Extraction and Preparation

(a) *Separation of propoxyphene salts from inert excipients in single component commercial dosage forms (e.g., Darvon, H03; Darvon-N, C53).*—Shake powder or 1/3 crushed tablet (after removing colored coating) with $CHCl_3$, filter through cotton or Whatman No. 1 paper to exclude excipient insolubles, and then evaporate on a steam bath until crystals form.

(b) *Preparation of propoxyphene base from propoxyphene salts or from propoxyphene–acetaminophen 2-component commercial dosage forms.*—Shake powder or 1/3 crushed tablet (after removing colored coating) in about 60 times its weight of water, make solution basic (pH 8) with $NaHCO_3$ and extract with an equal volume of hexanes. Evaporate hexanes layer to dryness in 60–65°C water bath to obtain base crystals.

(c) *Preparation of propoxyphene base from propoxyphene–aspirin, propoxyphene–APC multicomponent commercial dosage forms, or from illicit powders.*—(E. L. Gundy and A. E. Kemppainen, Michigan State Police Laboratory, Holland, MI, 1979, private communication.) Shake ca 300 mg powder or 1/3 crushed tablet (after removing colored coating) in ca 25 mL 0.5N HCl, wash with three 25 mL portions of benzene (or use 0.5N H_2SO_4 and $CHCl_3$ to avoid use of benzene), filter aqueous solution through Whatman No. 1 paper, make solution basic (pH 8) with $NaHCO_3$, and extract with an equal volume of hexanes. Evaporate hexanes layer to dryness in 60–65°C water bath to obtain base crystals.

(d) *Preparation of propoxyphene.HCl from base.*—Dissolve propoxyphene base in hexanes (or retain hexanes layer from previous extraction), bubble HCl vapors through hexanes layer to precipitate hydrochloride salt crystals, centrifuge, discard hexanes layer, wash twice with hexanes, and dry at 100–120°C to separate crystals from oily uncrystallized form.

(e) *Recommendations to aid in crystallization.*—Evaporate α-*d*-, α-*l*-, or β-propoxyphene base solutions in hexanes with heating below the melting point to crystallize. Heat oily, uncrystallized mother liquor that propoxyphene tends to form from solution to produce crystals. Cooling at 4°C crystallized the β-*dl*-propoxyphene base.

Microcrystal Tests

Place representative 0.1–0.2 mg portion (about the amount necessary to fill a small typewriter "o" without piling) of propoxyphene test sample powder or tablet contents on each of 3 clean microscope slides. To one of these slides add an equal amount of powdered sample standard of α-*d*-propoxyphene, to a second add a slightly greater amount (0.2–0.3 mg) of powdered sample standard of α-*l*-propoxyphene (e.g., Novrad, H14) and mix with clean glass rod. Add nothing to third slide. To fourth clean glass slide, add 0.1–0.2 mg portion of powdered sample standard of α-*d*-propoxyphene and 0.2–0.3 mg of powdered sample standard of α-*l*-propoxyphene and mix with clean glass rod. To samples on the 4 slides, add 1 drop of gold chloride microcrystal reagent solution, without stirring.

Let stand without cover slip for crystallization to occur. At intervals, examine each slide's drop contents under microscope (at 100–150×; then at ca 300× for confirmation) for formation of 15–50, μm elongated microcrystals, initially near edge. Under 100-150× magnification these appear tiny (length ca $1/100$–$1/30$ of the diameter of the field of view), in groups between amorphous precipitates and powder excipient particles. Disregard larger crystal formations (e.g., blades or needles). Note crystals formed and compare those on test sample slides with those formed on standard sample slide and with photomicrographs. Determine isomer present in test sample by comparison of microcrystals on 3 sample slides with those on standard sample slide.

Thermomicroscopy

(a) *Preparation of standard melting point crystals.*—Extract and prepare α-*d*- and α-*l*-propoxyphene base, hydrochloride, and napsylate crystals from commercial USP or NF dosage forms (e.g., Darvon, H03; Novrad, H14) according to procedures (a)–(d), above.

(b) *Hot stage calibration.*—Melt crystals of each Mettler Thermometric Standard substance on hot stage slide with cover glass according to prescribed procedures to calibrate digital scale readings over ambient to 300°C range.

(c) *Melting point measurements.*—Place a few crystals, 0.1 mg of test sample (obtained from HPLC or by alternative extraction procedures), on each of 3 clean glass hot stage slides. To 2 of these slides add, respectively, an approximately equal amount of the standard α-*d* and α-*l*-propoxyphene crystals of the same salt or base. Mix with glass rod, and label. Add cover glass to each of the 3 sample slides and gently crush by rotating with light pressure until crystals are just of distinguishable size under 100×–150× magnification. Replace each cover glass with a clean one. Successively place each of the 3 test sample slides in hot stage at ca 15–20°C below expected

Table 1. Melting points and eutectic melting points of isomers of propoxyphene bases and common salts

Compound	Melting point, °C		Eutectic melting point, °C
	d-Isomer	*l*-Isomer	Mixed *d*- and *l*-isomers
α-Propoxyphene base	73–75	73–75	62–64
α-Propoxyphene.HCl	165–167	165–167	131–133[a]
α-Propoxyphene napsylate	158–160	158–160	141–143
β-Propoxyphene base	—	—	41–43[b]
β-Propoxyphene.HCl	—	—	sublimes 165–201[c]

[a] If recrystallized from solution, mp 163°–168°C.
[b] Apparent eutectic mp of recrystallized racemate.
[c] Final melting at 199°–201°C.

melting point or eutectic melting point, and heat at rate of 2°/min. Close to expected melting point change to slower heating rate of 0.2°/min. Observe crystals through microscope at 100×–150× and record melting point and mixture melting points. Compare melting points of test sample and one of the mixtures, and eutectic melting point (temperature of first melting) of other mixture with Table 1. Determine propoxyphene isomer present in test sample as that complementary to standard isomer in eutectic melting mixture.

Results and Discussion

HPLC Separations and Identification

In the mobile phase medium, the UV absorption spectrum of propoxyphene shows 3 major peaks, at 251, 257, and 264 nm. The identification of standard propoxyphene and the propoxyphene present in the commercial and illicit preparations was based on the retention times and the ratios of the absorbance peak heights at 254 and 257 nm (where 254 nm is a fixed wavelength on one UV absorbance detector). The selection of 257 nm on the other UV-visible detector was due to the maximum absorbance at this wavelength. The absorbance ratios of α- and β-propoxyphene isomers lie in the range 0.91–0.92. The absorbance ratios of standard solutions of aspirin, phenacetin, caffeine, and acetaminophen are 1.45, 1.92, 0.91, and 1.48, respectively. These absorbance ratios, which supplement retention times as means of identification, can be measured with great accuracy and reproducibility (16). Additional identification parameters (other absorbance ratios) can be obtained by recording chromatograms at wavelengths other than 257 nm on the variable wavelength detector. This maximizes the probability of distinguishing propoxyphene from another compound in an unknown illicit sample, which may elute with the same retention time (17).

Two organic solvents, methanol or acetonitrile, mixed with the appropriate buffer at pH 7, were used to achieve baseline separations of propoxyphene hydrochloride or napsylate from various incipients (aspirin, caffeine, phenacetin, and acetaminophen) present in the commercial pharmaceutical preparations, and of the α and β diastereomers as well. The reverse phase columns used differed only in the particle size of the packing, 5 μm (Supelcosil LC-18) or 10 μm (μBondapak C_{18}). The results of all the separations under conditions studied in this investigation are summarized in Table 2. All of the incipients elute very close to void volume and thus are well separated from propoxyphene. As expected, the optical isomers of α- and the optical isomers of β-propoxyphene elute with the same respective retention times. Figures 1 and 2 are representative chromatograms obtained by the use of the 2 different mobile phases The tetrabutylammonium phosphate solution in the mobile phase could be replaced by ammonium phosphate at the same pH with essentially no difference in resolution or retention time (Table 2). The composition of the mobile phases c_1 and c_2 used with μBondapak C_{18} are quite similar to a_2 and a_1, respectively, used with Supelcosil LC-18. The corresponding retention times with these reverse phase columns are in the same range, and show the same trend of analytical separations.

Applied only to a crude synthetic mixture, Souter (18) reported chromatographic separation of α and β diastereomers using a Varian Micro-Pak NH_2 10 μm (weak ion exchanger) column with a mobile phase of 30% diethylamine in hexane. However, the elution pattern of these diastereomers is the same as has been found in the present investigation using reverse phase columns.

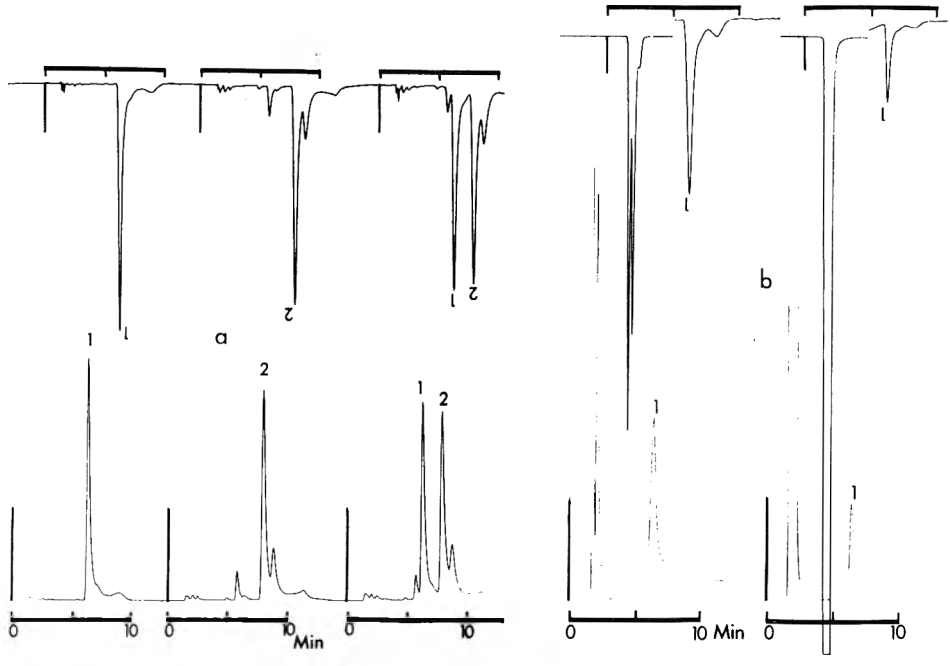

Figure 1. (a) Separation of standard α and β diastereomers: α-*d*-propoxyphene (1), β-*dl*-propoxyphene (2), and the mixture. (b) Chromatograms of Darvon Compound-65 in aqueous solution (left) and Darvocet-N 50 in methanol (right); AUFS = 1 for first 5 min. Mobile phase 68% acetonitrile with tetrabutylammonium phosphate buffer (pH 7); Supelcosil LC-18 column.

Thermomicroscopy

The use of a microscope stage that can be precisely heated for the characterization and identification of drugs, including α-propoxyphene.HCl, has been described by Kuhnert-Brandstatter (19) and McCrone (20). Eutectic melting points of binary mixtures of drugs with known substances are used in standard analytical identification procedures (21). In this investigation, the binary eutectic melting points of racemic mixtures of the optical isomers of α-propoxyphene base and common salts have been measured to differentiate the individual optical isomers. The melting points of the individual

Table 2. Retention times (min) on 2 columns of standard propoxyphene diastereomers and of α-*d*-propoxyphene and excipients present in pharmaceutical preparations

	Supelcosil LC-18, 5 μm, 150 × 4.6 mm id column				μBondapak C_{18}, 10 μm, 300 × 3.9 mm id column	
Sample	Solv. a_1 [a]	Solv. a_2 [a]	Solv. b_1 [b]	Solv. b_2 [b]	Solv. c_1 [c]	Solv. c_2 [c]
α-*d*-Propoxyphene	10.3	6.3	10.5	6.7	5.9	—
β-*dl*-Propoxyphene	11.9	8.0	12.4	8.2	6.9	—
Darvocet, C63						
α-*d*-Propoxyphene	10.4	6.4	10.5	6.7	6.0	—
Acetaminophen	2.8	1.8	2.2	2.0	2.5	—
Darvon, H06						
α-*d*-Propoxyphene	10.3	6.4	10.4	6.8	5.9	9.5
Aspirin, caffeine	1.9	1.7	2.0	1.9	1	2.1, 1.5
Phenacetin	2.4	2.1	2.4	2.2	1.8	2.8

[a] 0.01M solution of tetrabutylammonium phosphate (pH 7) with 75% methanol (a_1) or with 68% acetonitrile (a_2).
[b] 0.01M solution of ammonium phosphate (pH 7) with 75% methanol (b_1) or with 68% acetonitrile (b_2).
[c] 0.01M solution of tetrabutylammonium phosphate with 60% acetonitrile (c_1) or with a linear increasing concentration of methanol from 50 to 80% (c_2) at 5%/min.

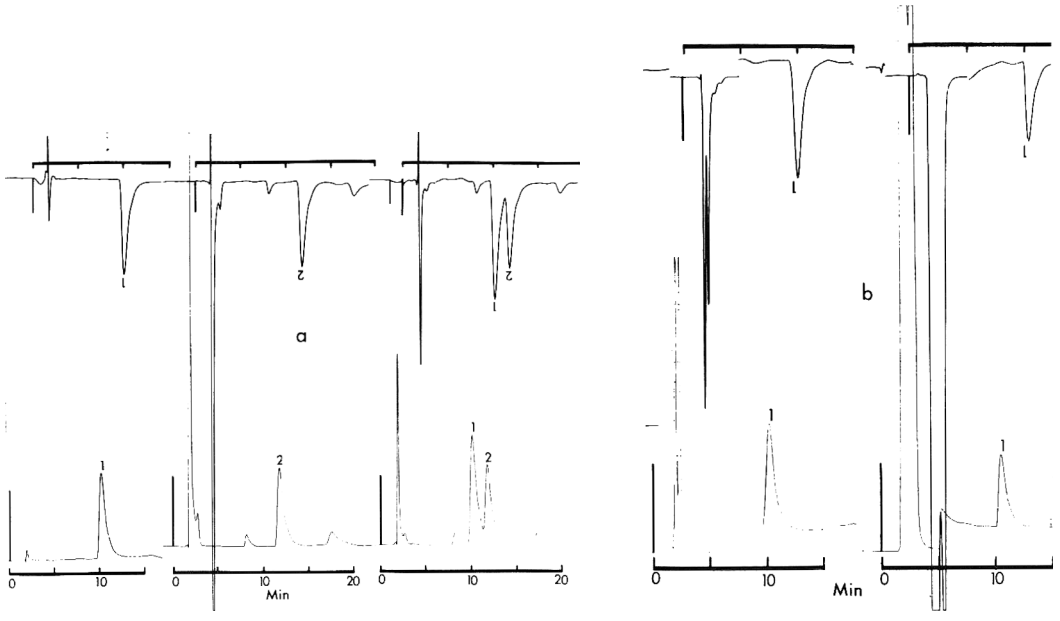

Figure 2. (a) Separation of standard α and β diastereomers: α-*d*-propoxyphene (1), β-*dl*-propoxyphene (2), and the mixture. (b) Chromatograms of Darvon Compound-65 in aqueous solution (left) and Darvocet-N 50 in methanol (right); AUFS = 1 for first 5 min. Mobile phase 75% methanol with tetrabutylammonium phosphate buffer (pH 7); Supelcosil LC-18 column.

optical isomers of the purified α-propoxyphene salts in available dosage forms have also been measured and agree with accepted values (22, 23). In addition, the melting behavior of β-*dl*-propoxyphene base and β-*dl*-propoxyphene.HCl has been studied to further characterize the disparity between the α- and β-propoxyphenes. The results are presented in Table 1. The mixtures of the 2 optical isomers of α-propoxyphene bases and salts form eutectics when prepared as described. The melting point of either isomer is depressed upon the addition of the other, so that melting begins at the eutectic temperature. Addition of the same isomer to a test sample isomer produces, of course, no change in the melting point. Standard crystals extracted and prepared from the commercial USP or NF propoxyphene standard samples listed served nicely as melting point standards with sufficient purity. The illicit propoxyphene-containing powders encountered by this laboratory have been successfully purified by HPLC elution or by the alternative extraction procedures outlined above, as revealed by the expected sharp melting points obtained.

The measured eutectic melting point of 131–133°C for α-*dl*-propoxyphene.HCl found in this investigation using a physical mixture of dry powders of the individual isomers as prescribed in the procedure is in the expected range for eutectic melting point depressions at these temperatures (19). Pohland and Sullivan (1) report the melting point of the racemic salt, α-*dl*-propoxyphene.HCl, recrystallized from solution at 170–171°C. The absence of a eutectic melting point was confirmed in this laboratory on a crystallized mixture of α-*d*- and α-*l*-propoxyphene.HCl, following their procedure. The formation of a racemic mixed crystal is thus indicated (24). The melting characteristics of such a solid solution depend on the precise isomer ratio in the recrystallized mixture. Our results show a range of melting points from 163° to 168°C, depending on the mixture ratio. The small difference between the mixed crystal melting point and the individual isomer melting point (165–167°C) may make the measurement of the former not analytically useful. The eutectic melting point of the dry mixture of the same isomers, as recommended in this study, is about 30° below the melting point of the individual isomers, and the melting depression is readily recognized.

A measurement of a eutectic melting point of β-*dl*-propoxyphene.HCl was inconclusive because the sample sublimed over the approximate

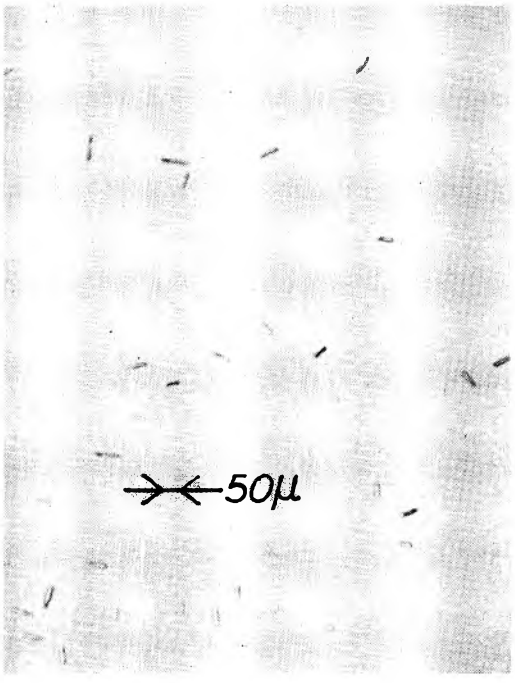

Figure 3. Photomicrograph (120×) of the characteristic *α-dl*-propoxyphene microcrystals in gold chloride reagent as they first appear.

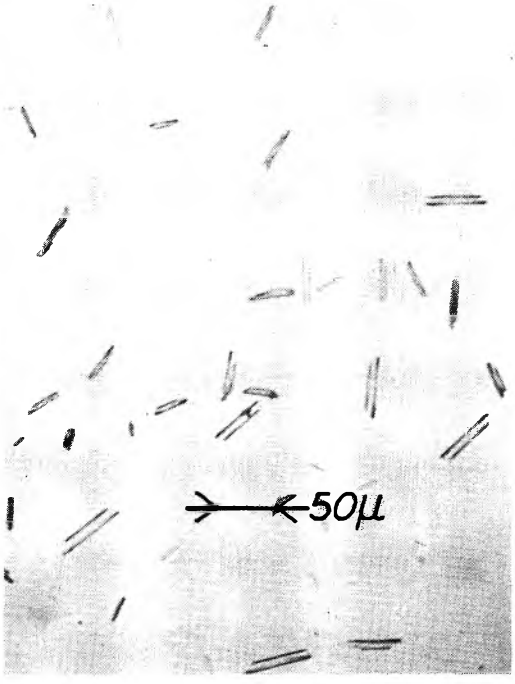

Figure 4. Photomicrograph (310×) of the characteristic *α-dl*-propoxyphene microcrystals in gold chloride reagent: typical early forms.

range 165–201°C. Final melting occurred at 201°C.

Microcrystal Tests

The microcrystal test for racemic α-propoxyphene has been applied to the crystals from the HPLC, commercial tablets, capsule contents, and to illicit powders comprised of the base or the different salts of the drug in combination with various adjuncts. Practical application of this test by laboratory chemists is often inhibited because the microscopic field of view is cluttered by excipients and amorphous precipitates, which mask the small characteristic microcrystals. The small quantity of powder material used for the test, permitted by its sensitivity, avoids cluttering the field of view. Extraction in chloroform (extraction procedure (**a**) above) may also be used. Fulton's gold chloride reagent formulation with glycerin was chosen to permit additional viewing time before evaporation. Figures 3–6 are presented to aid in the recognition of a positive test, the several specific shapes of microcrystals produced by *α-dl*-propoxyphene, as accepted by this laboratory.

Under 100–150× magnification these crystals first appear as tiny short thin rods, but higher

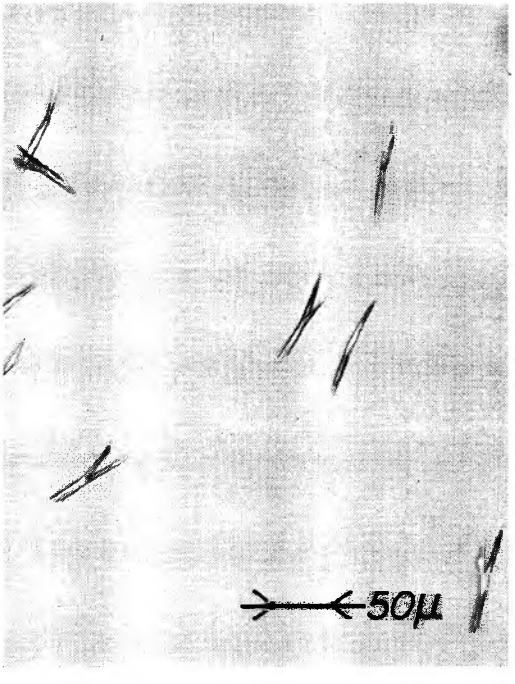

Figure 5. Photomicrograph (310×) of the characteristic *α-dl*-propoxyphene microcrystals in gold chloride reagent: typical mature forms.

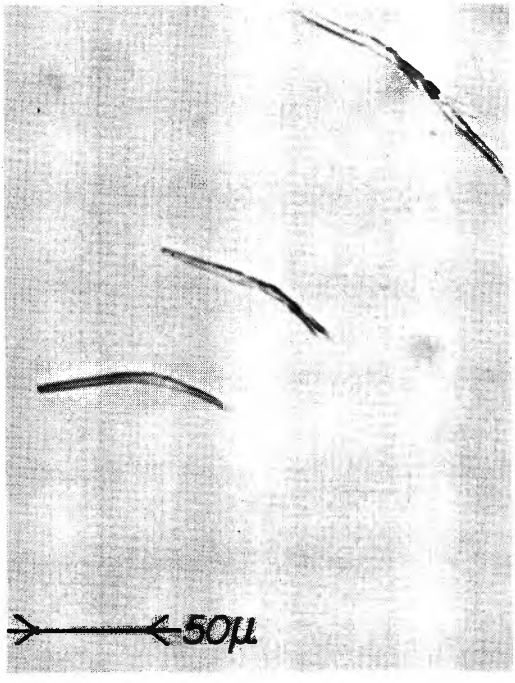

Figure 6. Photomicrograph (500×) of the characteristic α-*dl*-propoxyphene microcrystals in gold chloride reagent: typical mature forms.

magnification or slightly longer growing time reveals the distinct forms. Each of the standard commercial preparations of propoxyphene listed under *Reagents*, which represent the presently available dosage forms, as well as other commercial brands of propoxyphene preparations (not listed) and unpurified illicit powders received by this laboratory, produced these characteristic racemic crystals when tested as described.

Microcrystals formed by caffeine (needles) or acetaminophen (opaque burrs) in propoxyphene preparations, as well as by the single alpha optical isomers alone (needles), are much larger and different in shape from those for racemic forms, and are rapidly distinguished. For the test, only microcrystals formed within about 30 min should be considered, because thereafter α-*d*-propoxyphene napsylate has sometimes produced a few scattered microcrystals resembling the racemic ones.

The fundamental comparison nature of the microcrystal test, as emphasized by Koles (25), is embodied in the testing procedures described. The test sample slide displaying the racemic microcrystals like those on the standard slide (of mixed α-*d*- and α-*l*-propoxyphene) is noted, along with the standard isomer added. A test sample containing one alpha isomer would produce the characteristic microcrystals of a racemic mixture only on the slide to which the other alpha isomer was added. Slight morphological differences in the racemic crystals caused by the age of the reagent or other factors would show up on the standard α-*dl*-propoxyphene slide as well as on the test sample slides with which it is to be compared.

The extension of this test to β-propoxyphene, not generally available for comparison purposes, appears to increase its specificity, and is therefore of interest to its users. In combination with either alpha isomer or by itself, our samples of pure β-*dl*-propoxyphene hydrochloride or base did not produce the characteristic racemic microcrystals with the gold chloride reagent. The formation of the characteristic racemic α-propoxyphene microcrystals on the addition of a standard α-isomer powder to an unknown isomer of propoxyphene therefore strongly implies that the unknown is the other alpha isomer, and not a beta isomer, i.e., that the test is specific for α-*dl*-propoxyphene.

Conclusions

The methodology described for differentiating and characterizing α-*d*-propoxyphene in small samples is simple and specific. It has been systematically applied to standard dosage forms representative of all of the mixtures of α-*d*-propoxyphene with and without agents and excipients presently available, as well as to the illicit powders received by this laboratory.

High pressure liquid chromatography was used to separate and characterize the alpha and beta diastereomers in the sample. The optical isomers of α-propoxyphene have been differentiated and characterized by 2 methods, thermomicroscopy and microcrystal tests. These procedures are expeditious when the standards are prepared beforehand.

The procedures described applied to a test sample to identify the diastereomers present collectively contribute significantly to the characterization of propoxyphene itself. It appears very unlikely that another substance would be found that produces the same results as α-*d*-propoxyphene. Nevertheless, studies are continuing in this laboratory on GC–MS and further thermomicroscopic characterization of propoxyphene.

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Mass Spectral Quantitation of Cocaine HCl in Powders

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Gas-liquid chromatography (GLC) using a mass spectrometer (MS) as a detector is used for the quantitative determination of cocaine HCl in powders. After the sample is dissolved in methanol containing deuterated cocaine HCl internal standard, an aliquot is injected into the GLC-MS system with the mass spectrometer operating in the scan mode. The cocaine HCl concentration is calculated from the 303/308 m/e ratio. The procedure has a relative deviation of approximately 2.3% and is applicable to samples containing a wide variety of diluents and adulterants.

The use of a mass spectrometer (MS) as a sensitive detector in gas-liquid chromatography (GLC) has been increasing in forensic laboratories in recent years. The mass spectrometer has excellent sensitivity, and the spectrum produced is usually sufficient for the identification of the eluted compound. Under certain conditions the mass spectrometer can also be used to give quantitative information.

Abused substances which have been quantitated by GLC-MS include phencyclidine (1, 2), amphetamine (3, 4), codeine and morphine (5), tetrahydrocannabinol (6, 7), and heroin (8). The procedures for the quantitation of all these substances used their respective stable isotope-labeled counterparts as internal standards. Stable isotope-labeled molecules are probably the closest approach to an ideal internal standard. They have chemical and physical properties nearly identical to their nondeuterated counterparts, and will usually co-elute from the GLC column. They are distinguished by the fact that their molecular ions in the combined mass spectrum will differ in mass by the number of deuterium atoms in the stable isotope-labeled molecule. All the above procedures also used MS in the selective ion monitoring mode: The mass spectrometer is used as a detector for selected ions as opposed to scanning a given m/e range. Sensitivity is high with selective ion monitoring because computerized MS effectively uses time looking at only a few ions. However, much qualitative information is lost because only a few mass fragments are examined. For identification, it is desirable to examine the entire mass spectrum. The purpose of this study was to determine the feasibility of using GLC-MS operating in the scan mode for quantitation of cocaine in forensic samples.

Experimental

Instruments

Analyses were performed with a Finnigan MS Model 3300. Data were acquired and manipulated by standard software routines. The mass spectrum was scanned from mass 75 to mass 310. Masses 75 to 270 were counted for 3 ms each. Masses 271 to 310 were counted for 20 ms each. A new scan was initiated every 2 s. The ion source was operated at ambient temperature, at 70 V. The Finnigan Model 9500 gas chromatograph was equipped with a 2 mm id × 120 cm glass column containing 3% OV-1 on 100–120 mesh Gas-Chrom Q. The helium carrier gas flow rate was 40 mL/min. The injection port and column temperatures were 240 and 190°C, respectively. The 2 instruments were interfaced with a jet separator maintained at 210°C.

To determine the accuracy of the procedure, the same samples were analyzed on a Packard Model 873 GLC instrument using a previously collaborated procedure (9). The instrument was equipped with a hydrogen flame ionization detector, a column similar to that used in the GLC-MS studies, and a Columbia Scientific Industries integrator.

Preparation and Analysis of d_5-Cocaine HCl

Ecgonine was obtained by the acid hydrolysis of cocaine. The ecgonine was methylated with BF_3 in methanol. Deuterated benzene was monobrominated and then converted to a Grignard reagent using Mg and ether. The Grignard reagent was poured into dry ice and acid hydrolyzed to yield d_5-benzoic acid. The d_5-benzoic acid was converted to d_5-benzoyl chloride by reaction with thionyl chloride. Pentadeuterated cocaine was then produced by the reaction of the d_5-benzoyl chloride with the methyl ester of

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Table 1. Approximate composition of samples used

Sample	Composition	
	Compound	%
1	Cocaine HCl	80
	Inositol	
2	Cocaine HCl	68
	Lidocaine HCL	
3	Cocaine HCl	70
	Lidocaine HCl	
4	Cocaine HCl	85
5	Cocaine HCl	29
	Inositol	
	Lactose	
6	Cocaine HCl	33
	Lidocaine HCl	
	Inositol	
7	Cocaine HCl	80
	Inositol	
8	Cocaine HCl	87
9	Cocaine HCl	59
	Antipyrine	
10	Cocaine HCl	21
	Lidocaine HCl	
	Boric Acid	
11	Cocaine HCl	93
12	Cocaine HCl	90
13	Cocaine HCl	77
	Mannitol	

ecgonine. Analysis of the d_5-cocaine HCl, using GLC-MS selective ion monitoring, shows this material to be 99.7% isotopically pure. A conventional GLC procedure (9) indicated that the material was 97% cocaine (calculated as d_5-cocaine HCl); the remainder was the methyl ester of ecgonine. Stability studies indicate that methanol solutions of this material are stable at least 1 month at room temperature. The dry d_5-cocaine HCl is stable at least 1 year when stored in closed containers.

Preparation and Analysis of Samples

An amount of sample estimated to contain 100 mg cocaine HCl was transferred to a 250 mL volumetric flask and diluted to volume with methanol. Ten mL of this solution and 10 mL of methanol solution containing 0.4 mg/mL d_5-cocaine HCl were then added to a 25 mL volumetric flask and diluted to volume with methanol. A 0.2 μL aliquot was then injected into the GLC-MS system under the conditions previously described. A similar amount of standard cocaine HCl was identically treated. The amount of cocaine HCl in the sample was calculated from the 303/308 m/e ratio of the sample divided by that of the standard cocaine HCl. The scan at the top of the chromatographic peak was chosen to calculate these ratios.

Table 2. Comparative results for determination of cocaine HCl by 2 procedures

Sample	GLC, %	MS, %
1	79.8	81.5
2	68.0	68.5
3	69.9	75.4
4	84.6	78.8
5	28.7	27.3
6	33.0	33.1
7	79.9	76.2
8	86.7	89.3
9	58.7	58.8
10	21.0	20.6
11	92.6	93.6
12	90.4	89.6
13	77.4	75.7

Results and Discussion

Thirteen samples submitted as drug exhibits to this laboratory were analyzed by the 2 procedures. Table 1 lists the approximate composition of the samples. Table 2 lists the results obtained. A statistical technique using paired differences and the *t*-distribution (10) does not show any difference in the accuracy of the 2 procedures at the 95% confidence level. One sample was analyzed repetitively using separate sample weighings to determine the precision of the GLC-MS procedure. Table 3 shows the results obtained. Also shown in Table 3 is the ratio 272/303 m/e obtained on the scan used for quantitation. Mass/charge 272 is the fragment resulting from the loss of ($-OCH_3$) while 303 m/e is the molecular ion of cocaine. This ratio should be constant, independent of the amount of cocaine present. By comparison with the corresponding ratio obtained on the standard cocaine scan, this ratio serves as an aid in identifying the cocaine in the sample. Statistical treatment of values obtained is included in Table 3.

Table 3. Statistical results for repetitive assays of a single sample for cocaine HCl

Analysis	Cocaine HCl, %	Ratio, 272/303
1	33.1	0.400
2	31.5	0.429
3	33.5	0.385
4	32.3	0.373
5	33.9	0.379
6	33.9	0.379
Av.	33.0	0.391
SD	0.77	0.016
RSD, %	2.33	4.1

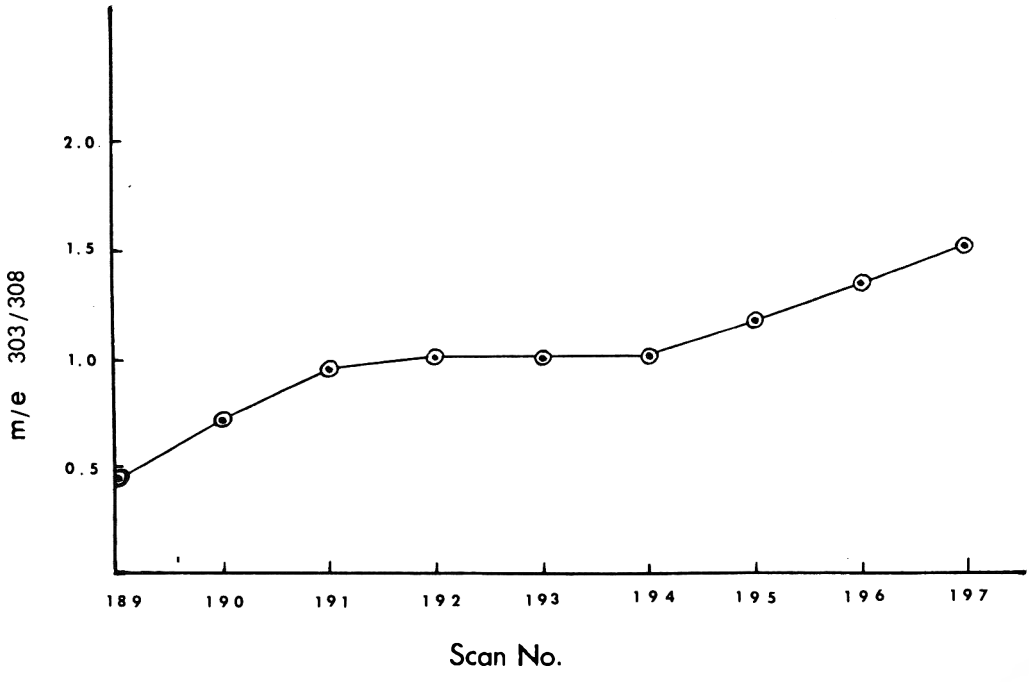

Figure 1. Ratio m/e 303/308 obtained on successive scans in a single chromatogram, indicating partial resolution of cocaine and d_5-cocaine. Chromatographic peak is at scan 193.

During the development of this procedure, it was noted that d_5-cocaine eluted about 5 s earlier than cocaine under the GLC conditions used. If they did co-elute, a constant ratio for 303/308 m/e, the molecular ions for cocaine and d_5-cocaine, respectively, would be obtained for all scans in the same chromatogram. Figure 1 shows that a constant ratio is not obtained. This GLC separation of a deuterated compound and its nondeuterated counterpart has also been noted for heroin (8). When a mass spectrometer is operated in the scan mode, this partial resolution could lead to errors in quantitation if the concentrations of d_5-cocaine and cocaine were considerably different. The top of the GLC peak, where the scan for quantitation is taken, will not be centered between the 2 unresolved peaks but will be shifted slightly toward the more con-

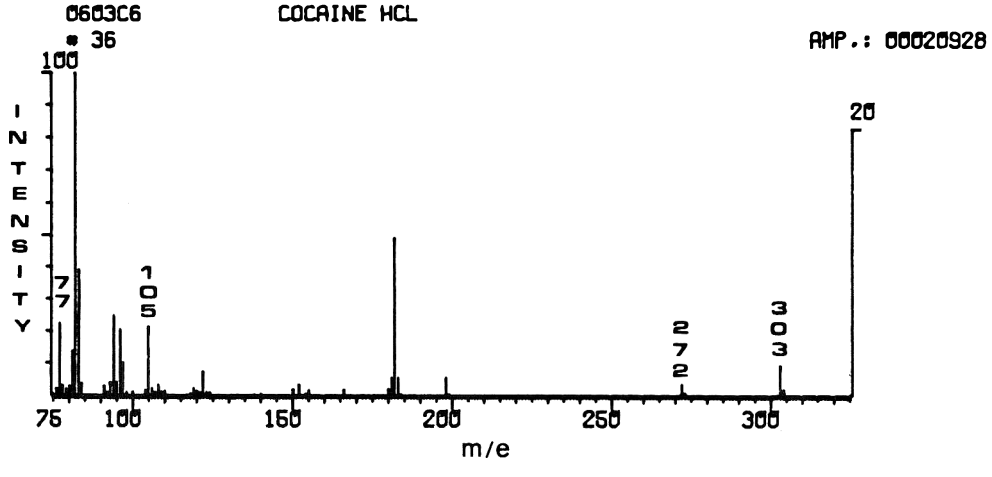

Figure 2. Mass spectrum of cocaine HCl showing fragments not present in spectrum of d_5-cocaine HCl.

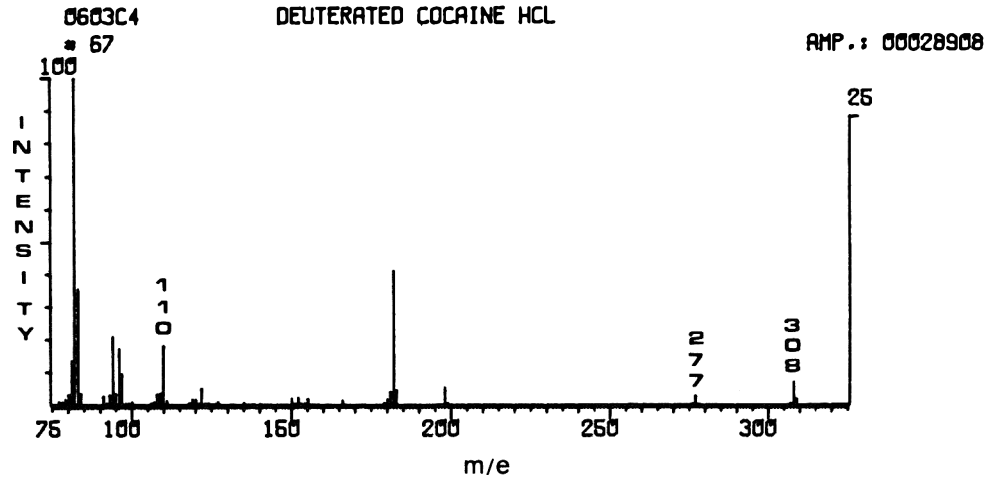

Figure 3. Mass spectrum of d_5-cocaine HCl showing fragments not present in spectrum of cocaine HCl.

centrated component. Thus, if the cocaine concentration is higher in the sample solution than in the standard solution, the assay result will be too high. If it is lower, the assay result will be too low. However, if the sample concentration can be approximated to within 50–200% of the standard cocaine concentration, little error will result. The 303/308 m/e response ratio is nearly linear over this concentration range, 0.016–0.064 μg cocaine HCl injected. Fortunately, the concentration of cocaine in a sample can usually be estimated to within the required accuracy by a simple microscopic examination of the sample. Sugars, if present, are easily recognized using 1 + 4 acetic acid–water mounting solution. The addition of a drop of 5% gold chloride in water to the slide will reveal the presence of lidocaine, a common cocaine adulterant. A satisfactory estimation of the sample cocaine content can be made by assuming the sample to be 50% cocaine if either sugars or lidocaine is present and 25% if both are present.

The advantage of a whole scan over the more accurate and more sensitive selective ion monitoring is that additional qualitative information is obtained. The mass spectrum obtained is a composite of the individual spectra of cocaine and d_5-cocaine. However, certain fragments

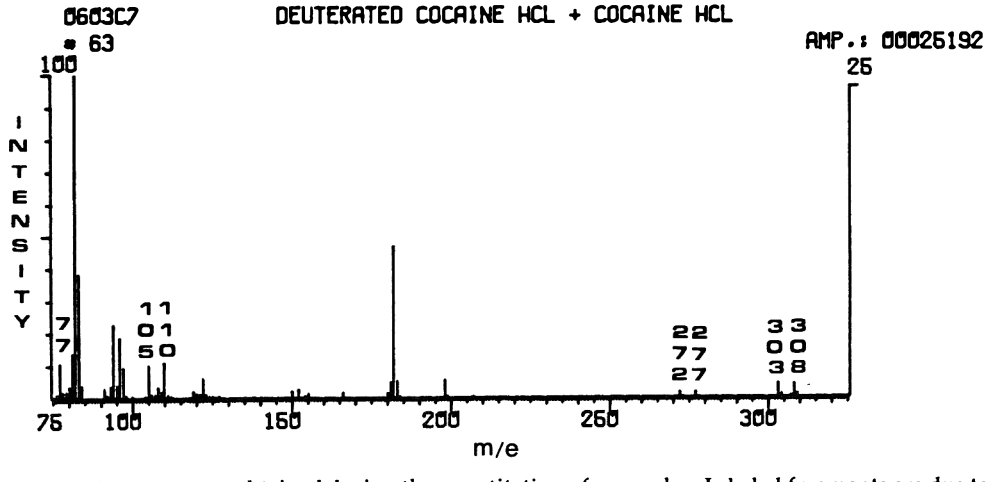

Figure 4. Mass spectrum obtained during the quantitation of a sample. Labeled fragments are due to individual components of the mixture.

present in the mass spectrum of cocaine but not in that of d_5-cocaine can be examined. Examples of these are m/e 77 (phenyl) and m/e 105 (benzoyl), present in relatively equal intensities. The absence of mass fragments not present in either cocaine or d_5-cocaine is also significant in the identification of cocaine in the sample. Figures 2–4 show mass spectra of cocaine, d_5-cocaine, and a combination of the two, respectively. Thus, to be mistakenly identified as cocaine, a compound would have to very nearly co-elute with d_5-cocaine, have all mass fragments present in the mass spectrum of cocaine but absent in the mass spectrum of d_5-cocaine in the proper ratios, and contain no mass fragments not present in either cocaine or d_5-cocaine.

Although the standard deviation of this procedure for the MS quantitation of cocaine is approximately 3 times that of a collaborated GLC procedure (9), it has the advantage of minimal sample preparation time and good specificity for cocaine.

It is recommended that study continue. Additional tests would need to be done on the sample to confirm that the cocaine present is levorotatory.

Acknowledgment

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High Performance Liquid Chromatographic Determination of Methapyrilene Hydrochloride in Feed and Sleep Aid Tablets

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Methapyrilene hydrochloride (MP·HCl) was extracted from feed with methanol and determined by reverse phase partition chromatography in less than 15 min, using isocratic elution with acetonitrile–1.1% ammonium carbonate (1 + 1) as the mobile phase. This procedure was tested on feed treated with MP·HCl at levels of 125, 500, and 2000 ppm. Recoveries were 104, 95, and 96% with coefficients of variation of 2.4, 1.6, and 0.6%, respectively. MP·HCl in feed was stable for 14 days. This method was also successfully used to determine MP·HCl in 3 sleep aid tablets.

High performance liquid chromatography (HPLC) has been increasingly used in the analysis of animal feed. Some recent examples include vitamin E (1) and 2-acetylaminofluorene (2) in treated feed, and various drugs (3–5) in medicated feed.

Methapyrilene hydrochloride (MP·HCl), an antihistamine, is one of the active ingredients in a number of pharmaceutical preparations. These preparations are usually analyzed spectrophotometrically (6, 7). Since MP·HCl is a common drug ingredient, its potential for carcinogenicity was being tested by feeding it to rats (8). The determination of MP·HCl in feed has not been reported; therefore, a rapid quantitative method was developed for measuring the drug and its stability in the feed used in the rat study. The method involves a simple methanol extraction of the MP·HCl–feed mixture and injection of an aliquot of the extract into an HPLC column. Precise and accurate quantitation of MP·HCl was achieved by using valerophenone as an internal standard. Use of the method was extended to include sleep aid tablets because MP·HCl is a common ingredient in many over-the-counter sleep aids.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—HPLC system consisted of: Laboratory Data Control Constametric II pump (Riviera Beach, FL 33404), Chromatronix dual-channel ultraviolet (UV) absorbance detector (Spectra-Physics, Santa Clara, CA 95051), high pressure Rheodyne Model 7120 sample injector equipped with 100 μL sample loop (Rheodyne, Inc., Berkeley, CA 94710), and Fisher Recordall Series 5000 recorder (Fisher Scientific Co., Pittsburgh, PA 15219) operated at 0.2 in./min chart speed and 10 mV setting. Peak areas and retention times were determined with Hewlett-Packard (Avondale, PA 19311) 3352A laboratory data system.

(b) *Analytical column.*—30 cm × 3.9 mm id stainless steel, packed with μBondapak® C_{18} (Waters Associates, Milford, MA 01757). The column was flushed with acetonitrile–water (1 + 1) at the end of each day.

(c) *Solvents.*—Distilled-in-Glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442) organic solvents and deionized water were used throughout the study.

(d) *Mobile phase.*—Acetonitrile–1.1% ammonium carbonate (J.T. Baker Chemical Co., Phillipsburg, NJ 08865) (1 + 1) at a flow rate of 2 mL/min was used. The 1.1% ammonium carbonate solution was filtered through a Millipore solvent clarification apparatus (Millipore Corp., Bedford, MA 01730) before mobile phase preparation. The mobile phase was degassed daily in an ultrasonic bath under vacuum before use.

(e) *Standard solutions.*—5, 50, 250, 375, and 500 μg MP·HCl/mL methanol. To 2 mL of each standard solution was added 25 μL internal standard (f). Injections of 30 μL from the former 2 solutions and 10 μL from the latter 3 solutions were made to prepare a calibration curve. Another calibration curve using MP·HCl standards extracted from blank feed consisted of similar concentration and injection volumes. However, 30 mL methanol was used to extract spiked feed to give concentrations of 5 and 50 μg/mL and 75 mL methanol to give 250, 375, and 500 μg MP·HCl/mL. The extraction procedure used is described below (*Preparation of Sample and Extraction Procedure*).

Stock solutions.—Three stock solutions (A, B, and C) of MP·HCl (Sigma Chemical Co., St. Louis,

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MO 63178) were prepared in glass-distilled methanol and stored at 5°C until used. Solutions A, B, and C (0.25, 0.50, and 4.0 mg/mL) were used for the recovery experiments at 125, 500, and 2000 ppm, respectively.

(f) *Internal standard.*—1 mL valerophenone (Aldrich Chemical Co., Milwaukee, WI 53233) was diluted to 100 mL with methanol (9.88 mg/mL). The solution was stored in the cold.

Preparation of Sample and Extraction Procedure

Ten g feed samples were weighed into separate tared serum bottles, and MP·HCl stock solutions were added to give concentrations of 125, 500, and 2000 ppm. Methanol was added to bring the total volume to 30 mL for 125 and 500 ppm and 75 mL for 2000 ppm. MP·HCl was extracted from the feed by shaking 30 min on an Eberbach shaker (Eberbach Corp., Ann Arbor, MI 48106). After the sample stood 30 min to let coarse particles settle, a 5 mL aliquot of the methanol supernate was refrigerated overnight to let the fine particles settle. Then 2 mL supernate was transferred to another vial and 25 μL internal standard was added. After vortexing, the solution was analyzed by HPLC. This extraction procedure was also used for the preparation of samples for the calibration curve consisting of MP·HCl standards extracted from blank feed.

To facilitate the removal of the fine particles from the extract, 3 techniques were examined. The extract was (a) centrifuged 10 min with a table-top centrifuge, (b) filtered with a Waters Associates organic sample clarification kit, and (c) filtered through 2 μm pipet tip filters (Supelco, Bellefonte, PA 16823). All of these procedures, including overnight settling of fine particles, were satisfactory. The last technique, however, was the least time-consuming and most convenient for routine work.

Tablets representing 3 brands of sleep aid preparations (purchased from a local drug store) were dissolved separately in 25 mL methanol, vigorously shaken, and filtered through 2 μm tip filters. An aliquot of each filtered extract was directly injected into the HPLC column. The tablets, when dissolved in methanol, gave blue, pink, and colorless solutions, respectively, and contained some undissolved particles. Only 1 tablet (blue solution) contained another active ingredient, scopolamine aminoxide hydrobromide, as declared on the bottle label.

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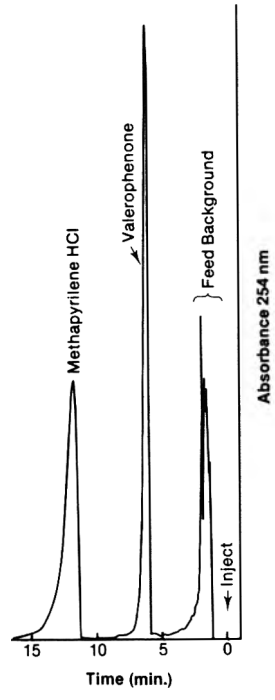

Figure 1. HPLC chromatogram of 30 μL 125 ppm MP·HCl in feed extract. Conditions: column μBondapak C_{18}, mobile phase acetonitrile-1.1% ammonium carbonate (1 + 1), detector UV 254 nm, sensitivity 0.16 absorbance unit full scale, flow rate 2.0 mL/min, chart speed 0.2 in./min.

Stability of MP·HCl in Feed

Five kg feed was transferred to a V-shaped blender and MP·HCl (dry powder) was added to give the desired concentration (ppm level). The chemical–feed mixture was blended 15 min, followed by withdrawal of ca 300 g for the stability study. Three 10 g samples were weighed from the 300 g on days 0, 2, 7, and 14. These samples were carried through the extraction and HPLC procedures described above for the determination of MP·HCl.

Results and Discussion

Figure 1 shows a chromatogram of a 30 μL sample of feed extract spiked with 125 ppm MP·HCl. The first peak represents background compounds in the feed, the second the internal standard valerophenone, and the third MP·HCl. Retention time, peak area, and peak shape were all highly reproducible under the experimental conditions used. The average retention times for 7 replicate injections of MP·HCl and valerophenone were 11.2 ± 0.5 and 5.9 ± 0.2 min (average

Table 1. Recovery of MP·HCl from feed

Added, ppm	Found, ppm	Rec., %
125	127	101.6
125	130	104.0
125	132	105.6
Av. ± CV[a]	129.7 ± 1.9	103.7 ± 2.4
500	479	95.9
500	470	94.0
500	479	95.9
Av. ± CV	476 ± 1.5	95.3 ± 1.6
2000	1927	96.4
2000	1905	95.3
2000	1912	95.6
Av. ± CV	1915 ± 0.6	96.1 ± 0.6

[a] Coefficient of variation, %.

± standard deviation), respectively. A calibration curve, using valerophenone as an internal standard and 0.15, 1.5, 2.5, 3.75, and 5 μg MP·HCl, was linear, with a correlation coefficient of 0.999. Linearity was also achieved (correlation coefficient 0.999) with MP·HCl standards extracted from blank feed. Use of this curve eliminated the need for recovery corrections in the determination of MP·HCl in feed. The least square lines for standards nonextracted and extracted from blank feed were $y = 0.010455x - 0.013900$ and $y = 0.009557x - 0.01208$, respectively. Since the intercepts are similar, the ratio of slopes of extracted vs nonextracted standards can be used to determine absolute recoveries: recovery = $(0.009557/0.010455) \times 100 = 91.4\%$. Therefore, the absolute recoveries would be 91.4% of the recoveries obtained with the standard curve using standards extracted from blank feed.

Recovery experiments were carried out on feed preparations formulated to contain 125, 500, and 2000 ppm MP·HCl (Table 1). The average recoveries were 104, 95, and 96%, with coefficients of variation of 2.4, 1.6, and 0.6%, respectively, indicating good precision.

The stability of MP·HCl in feed at the 125 and 2000 ppm levels over 14 days was determined. No significant degradation of MP·HCl in feed was observed (Table 2). The recovery data in Table 2 further illustrate the accuracy of the method.

This assay procedure was used to determine MP·HCl in 3 sleep aid products. All 3 tablets assayed higher (26.2, 26.0, and 28.2 mg or 106, 104, and 113%) than the label declaration of 25 mg MP·HCl/tablet, but the values were still within a reasonable range.

Kikita and Stange (9) reported the use of a series of stable internal standards for many HPLC systems leading to improved quantitation. In our study, valerophenone was selected as the internal standard because it gave a peak which was completely resolved from feed background and MP·HCl peaks. The calibration curve using an internal standard was used over a period of about 8 months with good recoveries of MP·HCl in feed added at various levels.

This HPLC method was used routinely to monitor dose levels and homogeneity of MP·HCl in an animal testing program. The minimum amount of MP·HCl detected was 50 ng at a sensitivity setting of 0.02, UV wavelength of 254 nm, and a signal-to-noise ratio of about 5. The detection level could be improved to a few nanograms by using higher sensitivity settings and the optimum wavelength for MP·HCl of 238 nm.

An alternative mobile phase, acetonitrile–0.1N ammonium dihydrogen phosphate (1 + 1), was also tested. This solvent combination gave retention times, sensitivity, and a linear relationship similar to those obtained with the mobile phase normally used.

This method makes possible the simple, rapid, and accurate determination of MP·HCl in feed mixtures and demonstrates its application to the

Table 2. Determination of stability of MP·HCl in feed[a]

MP·HCl added, ppm	Day			
	0	2	7	14
125	127	131	128	125
125	131	127	129	124
125	132	128	130	123
Av. ± CV	130 ± 2	129 ± 1.6	129 ± 0.8	124 ± 0.8
2000	1890	1920	1940	2000
2000	1860	1960	1990	1940
2000	2060	2010	1990	2100
Av. ± CV	1940 ± 5.6	1960 ± 2.3	1973 ± 1.5	2013 ± 4.0

[a] MP·HCl found, ppm.

analysis of sleep aid tablets. Further work will be required to make this procedure specific for the analysis of various pharmaceutical products. The need for tedious cleanup, which usually accompanies feed analyses, is avoided by employing a simple one-step methanol extraction of MP·HCl-treated feed as well as tablets.

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MICROBIOLOGICAL METHODS

Pre-enrichment Broths for Recovery of *Salmonella* from Milk Chocolate and Edible Casein: Collaborative Study

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A collaborative study was conducted to compare the relative efficiency of nonfat dry milk with brilliant green dye (NFDM-BG) and buffered peptone water (BPW) as pre-enrichment broths for recovery of *Salmonella* from milk chocolate. Lactose broth and modified lactose broth with added 1% $NaHCO_3$ and brilliant green dye were compared as pre-enrichment broths for recovery of *Salmonella* from edible casein. Two sets of 8 samples each of milk chocolate, containing initial levels of *Salmonella* ranging from <0.03 to 43 organisms/g, were examined by 13 collaborators. Of 104 determinations, 102 (98.1%) and 100 (96.2%) using NFDM-BG and BPW, respectively, were in agreement with sample results of the control laboratory. Two sets of 7 samples each of edible casein, containing initial levels of *Salmonella* ranging from <0.03 to 93 organisms/g, were also examined by the 13 collaborators. Of 91 determinations, 87 (95.6%) and 88 (96.7%) using lactose broth and modified lactose broth, respectively, were in agreement with sample results of the control laboratory. For recovery of *Salmonella*, therefore, NFDM-BG pre-enrichment is recommended for milk chocolate, and lactose broth is recommended for casein. The proposed revision of official final action method 46.054-46.067 has been adopted official first action.

Because contamination of chocolate products with *Salmonella* has been a serious health problem, the Food and Drug Administration (FDA) has recently focused its attention on methodology for detecting this pathogen in chocolate. Wilson et al. (1) evaluated 6 pre-enrichment broths for recovering *Salmonella* from milk chocolate. In that study 2 pre-enrichment broths, buffered peptone water (BPW) or reconstituted nonfat dry milk with 0.002% brilliant green dye (NFDM-BG), gave the highest recovery of *Salmonella*. This collaborative study compared the relative efficiency of these 2 pre-enrichment broths for their relative efficiency in recovering *Salmonella* from milk chocolate.

Since the detention by FDA of several lots of casein contaminated with *Salmonella* from 1977 to 1980, attention has also been given to edible casein, which is a binding agent, used as an ingredient of chocolate and many other foods such as breads and cereals. No microbiological method of analysis for casein contaminated with *Salmonella* is presently specified in *Official Methods of Analysis* (2). Poelma et al. (3) evaluated 8 pre-enrichment broths for the recovery of *Salmonella* from edible casein. Of 6 pre-enrichment broths tested, most probable number (MPN) values with lactose broth were consistently high in artificially inoculated casein. Because most processed foods used in the isolation of *Salmonella* are pre-enriched in lactose broth, this broth was chosen for the collaborative study. A modification of lactose broth (added brilliant green dye and 1% $NaHCO_3$) was evaluated because it is similar to that recommended by the Dairy Laboratory, Department of Agriculture, Wallaceville, New Zealand (4) for the analysis of edible casein powder. This collaborative study was undertaken to compare the relative efficiency of lactose broth and modified lactose broth in recovering *Salmonella* from edible casein and to establish an AOAC method for this product.

Collaborative Study

Each of 14 participating laboratories was furnished with duplicate sets of 8 samples of milk chocolate and duplicate sets of 7 samples of edible casein (Table 1). For each set of milk chocolate samples, 3 of 4 samples of one brand of milk chocolate were inoculated with *Salmonella rubislaw*, and 3 of 4 samples of another brand of milk

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Table 1. Levels of *Salmonella* serotypes inoculated into samples of milk chocolate and edible casein

Product	Serotype	Inoculum dilution	Level	Most probable number/g			
				Day 0[a]		Day 14[b]	
				NFDM[c]	BPW[d]	NFDM	BPW
Milk chocolate (Brand A)	*S. rubislaw*	10^0	1	430	430	43	23
		10^{-1}	2	230	93	0.93	9.3
		10^{-2}	3	9.3	3.9	0.43	0.09
	uninoculated	none	4	<0.03	<0.03	<0.03	<0.03
Milk chocolate (Brand B)	*S. senftenberg*	10^0	1	23	43	4.3	0.43
		10^{-1}	2	2.3	0.93	0.23	<0.03
		10^{-2}	3	0.75	0.23	<0.03	0.04
	uninoculated	none	4	<0.03	<0.03	<0.03	<0.03
				L[e]	L (mod.)[f]	L	L (mod.)
Lactic casein	*S. infantis*	10^0	1	23 000	23	93	9.3
		10^{-1}	2	430	9.3	1.5	0.91
		10^{-2}	3	9.3	2.3	0.23	0.15
	uninoculated	none	4	<0.03	<0.03	<0.03	<0.03
Acid casein	*S. anatum*	10^0	1	230	9.3	2.1	0.93
		10^{-1}	2	23	2.3	0.43	0.09
	uninoculated	none	3	<0.03	<0.03	<0.03	<0.03

[a] Analyzed 1 day after inoculation.
[b] Analyzed 14 days after inoculation, when collaborative analyses were begun.
[c] Reconstituted nonfat dry milk with brilliant green dye (final concentration 0.002%).
[d] Buffered peptone water.
[e] Lactose broth.
[f] Lactose broth modified by addition of 1% $NaHCO_3$ and brilliant green dye (final concentration 0.002%).

chocolate were inoculated with *S. senftenberg*. For each set of casein samples, 3 of 4 samples of lactic casein were inoculated with *S. infantis*, and 2 of 3 samples of acid casein were inoculated with *S. anatum*.

Samples of chocolate and casein were inoculated as follows: A 10 mL volume of brain heart infusion broth inoculated from a stock culture was incubated for 24 ± 2 h at 35°C. The cells were harvested by centrifugation at 5000 rpm (3090 ×*g*) for 10 min, washed twice in Butterfield's phosphate buffer (pH 6.8 − 7.2), and resuspended in 10 mL buffer. Each of 2 brands of chocolate samples was inoculated with a different *Salmonella* serotype. Each of the 2 sets of these samples was inoculated with 1 mL undiluted (10^0), 10^{-1}, and 10^{-2} dilutions of washed cell suspension, representing samples at levels 1, 2, and 3, respectively. The fourth sample level, which was not inoculated with *Salmonella*, served as the negative sample control. For each set of lactic casein samples, 0.1 mL from the 10^0, 10^{-1}, and 10^{-2} dilutions of washed cell suspension was used to inoculate samples at levels 1, 2, and 3, respectively; 0.1 mL from the 10^0 and 10^{-1} dilutions of washed cell suspension was used to inoculate acid casein at levels 1 and 2, respectively. The fourth sample level of lactic casein and the third sample of acid casein, being negative sample controls, were not inoculated with *Salmonella*.

Retail packages of each brand of milk chocolate (chips) were pooled into a large sterile bag, thoroughly mixed, and dispensed into four 1600 g portions. Each portion was melted in a double boiler or in a steam bath, stirred, and allowed to equilibrate in a 60°C water bath. Each brand of milk chocolate was initially examined using methods described in this study to ensure the absence of any naturally occurring *Salmonella*. Each sample was then seeded with *Salmonella* cells at levels specified above. Duplicate sets of individual 30 g samples representing each sample level were prepared for each collaborator and the originating laboratory.

Samples of lactic casein and acid casein were prepared from large (55 lb) commercial bags of product. Both types of casein were initially examined using methods described in this study to ensure the absence of any naturally occurring *Salmonella*. Each type of casein powder was mixed in a Norton Jar Mill (Scientific Products, McGaw Park, IL 60085) with grinding media for 18–24 h and dispensed in 30 g portions into sterile plastic bags. The positive samples were seeded with 0.1 mL *Salmonella* cell suspensions,

at levels specified above. Duplicate sets of individual 30 g samples representing each sample level were prepared for each collaborator and the reference laboratory.

Samples for the collaborative study were stored at room temperature until they were shipped. Each sample was assigned a different sample number selected from a standard statistical table of random numbers (5). The 14 collaborators received duplicate sets of 8 samples of milk chocolate and duplicate sets of 7 samples of edible casein with final instructions for the collaborative method study. They were requested to store samples at room temperature until analysis, asked to initiate their examination on the same (specified) day, and instructed to report results for each sample as positive or negative for *Salmonella* on the standardized sheets furnished with samples.

The initiating or control laboratory performed a 3-tube MPN determination on the samples immediately after inoculation (day 0) and again 14 days after inoculation, the day when actual analyses were initiated by collaborators (Table 1). This study was performed in 2 segments: Samples of milk chocolate were prepared and shipped during the second week after preparation; the samples of casein were prepared 2 weeks later and shipped during the second week after preparation. Collaborators were asked to pre-enrich one of the duplicate sets of milk chocolate samples in reconstituted NFDM (final dye concentration 0.002%), and the corresponding set of replicate samples in BPW. They were also requested to pre-enrich one set of casein samples in lactose broth and the corresponding replicate set in modified lactose broth (final $NaHCO_3$ concentration 1%; final dye concentration 0.002%).

Method

All media and reagents used in this study were prepared as described in *Official Methods of Analysis* (2), **46.054-46.067,** with the following additions made:

(a) In the applicability statement, following ". . . dried egg white," add "edible casein, milk chocolate . . ."

(b) Add as a new section in **46.054:**

(**v**) *Reconstituted nonfat dry milk with brilliant green dye (NFDM-BG).*—Suspend 100 g dehydrated NFDM in 1 L H_2O; mix by swirling until dissolved. Autoclave 15 min at 121°. Add brilliant green dye soln, **46.055(n),** after blending sample/broth mixture as in **46.056(e).**

(c) Add as a new section in **46.056:**

(**e**) *Milk chocolate and casein.*—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile reconstituted NFDM, **46.054(v),** to chocolate samples, and add 225 mL lactose broth, **46.054(a),** to casein samples. Blend each sample/broth mixt. 2 min at high speed and decant blended homogenate into sterile 500 mL jar. Cap jar securely and let stand 60 min at room temp. Mix well by shaking, and det. pH with test paper, **46.055(l).** Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, **46.055(c)** or **(d),** capping jar securely and mixing well before detg final pH. To chocolate-reconstituted NFDM samples, add 0.45 mL 1% aq. brilliant green dye, **46.055(n),** and mix well. Loosen jar caps ¼ turn and incubate jar 24 ± 2 h at 35°.

The following media were also evaluated as part of the collaborative study:

(**a**) Buffered peptone water (BPW).—Suspend 10.0 g peptone, 5.0 g NaCl, 9.0 g $Na_2HPO_4.12H_2O$, 1.5 g KH_2PO_4 in 1 L H_2O; mix thoroughly. Heat to dissolve components; adjust pH to 7.2 ± 0.2. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2. Prepare medium 3–11 days before use.

(**b**) Modified lactose broth (lactose broth with added $NaHCO_3$ and brilliant green dye).—Dissolve 3.0 g beef extract, 5.0 g polypeptone or peptone, 5.0 g lactose, 10.0 g $NaHCO_3$ in 1 L H_2O. Autoclave 15 min at 121°C. Final pH, 8.0 ± 0.2. Blend sample/broth mixture for 2 min at high speed, decant blended homogenate into sterile jar, and let stand 60 min. Mix well by swirling; determine pH with test paper **46.055(l)** and adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, **46.055(c)** or **(d);** mix well before determining final pH. Add 0.45 mL 1% aqueous brilliant green dye, **46.055(n),** and mix well before incubating.

Results and Recommendations

The MPN levels of *Salmonella* in the collaborative samples of milk chocolate and casein immediately after inoculation (day 0) and 14 days later, when actual collaborative analyses were initiated, are shown in Table 1. The presence of antimicrobials (anthocyanin compounds) in cocoa (6, 7) may have limited the survival of *Salmonella* in this product, thus reducing the number of *Salmonella* cells originally added to the chocolate. The cell population also may have declined because of the heat shocking temperature (60°C) that the inocula received upon addition to the melted chocolate. Low moisture or low water activity (a_w) values in the dry powder may have caused the reduction of *Salmonella* population in the casein powder, a condition

Table 2. Comparative recovery of *Salmonella rubislaw* or *S. senftenberg* from milk chocolate pre-enriched in reconstituted nonfat dry milk with brilliant green dye (NFDM-BG) or buffered peptone water (BPW)

	Brand A *S. rubislaw* level[a]								Brand B *S. senftenberg* level[a]							
	1		2		3		4		1		2		3		4	
Coll.	NFDM[b]	BPW	NFDM	BPW	NFDM	BPW	NFDM	BPW	NFDM	BPW	NFDM	BPW	NFDM	BPW	NFDM	BPW
1	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
2	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
3	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
4	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
5[c]	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−
7	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
8	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
9	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
10	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
11	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−
12[d]	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
13	+	+	+	+	+	+	−	−	+	+	+	+	−	−	−	−
14	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
Control	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
	In Agreement with Control Laboratory[e]															
No.	13	13	13	13	13	13	13	13	13	13	13	13	12	10	12	12
%	100	100	100	100	100	100	100	100	100	100	100	100	92	77	92	92

[a] Number of *Salmonella* cells per level given in Table 1.
[b] Sample pre-enriched in reconstituted NFDM with brilliant green dye (final concentration 0.002%).
[c] Initiated analysis 1 day late.
[d] Results of Collaborator 12 were excluded from analysis; collaborator neglected to use both selective broths.
[e] Evaluation based on a total of 13 collaborators.

which could cause osmotic shock to the cells when the samples were rehydrated. Even though the 3 inoculation levels represent inocula of serial 10-fold dilutions of the original washed cell suspension, a 10-fold difference was not observed among the corresponding sample levels analyzed at day 0 or day 14. This variation in sample population may be due to the interrelation of temperature, pH, water activity, and chemical constituents of the food products.

Table 2 shows the relative recovery of *S. rubislaw* and *S. senftenberg* from milk chocolate pre-enriched in reconstituted NFDM-BG or BPW. The control laboratory confirmed the presence of *Salmonella* in the inoculated samples at levels 1–3 and the absence of *Salmonella* in the uninoculated samples at level 4. Collaborator 5 initiated analysis 1 day late; however, recovery of *Salmonella* from the 3 inoculated levels of brand B was not significantly affected. This analyst reported positive results in both pre-enrichment broths for uninoculated samples, level 4. The results from Collaborator 12 listed in this table were excluded from further consideration in this study because of the analyst's failure to use both selective broths as specified in the methods for the collaborative study. Therefore, comparisons of the relative efficiency of these 2 pre-enrichment media for detecting *Salmonella* were made on 39 positive samples and 13 negative samples for each of 2 brands of milk chocolate. In 39 (100%) of the comparisons of brand A, *S. rubislaw* was detected in the 2 replicate samples that were pre-enriched in each of the 2 nonselective broths. All uninoculated comparisons were reported as *Salmonella*-negative. In comparisons of brand B milk chocolate inoculated with *S. senftenberg*, 38 of 39 (97.4%) samples pre-enriched with NFDM-BG were positive and 36 of 39 (92.3%) of those pre-enriched with BPW were positive. In 2 (5.1%) of the comparisons, *Salmonella* was detected in samples pre-enriched in NFDM-BG but not in replicate samples pre-enriched in BPW. In 1 (2.6%) of the sample comparisons, *Salmonella* was not detected in the 2 replicate samples pre-enriched by NFDM-BG or BPW. Collaborator 13 probably did not detect *Salmonella* in level 3 because of the non-uniform distribution of low numbers of viable *Salmonella* cells in those samples. The originating laboratory established the number of viable *S. senftenberg* cells in level 3 at <0.03/g and 0.04/g, respectively, using

Table 3. Comparative recovery of *Salmonella infantis* or *S. anatum* from edible casein pre-enriched in lactose broth (L) or modified lactose broth (L-M)

	Lactic casein *S. infantis* level[a]								Acid casein *S. anatum* level[a]					
	1		2		3		4		1		2		3	
Coll.	L	L-M[b]	L	L-M	L	L-M	L	L-M	L	L-M	L	L-M	L	L-M
1	−	+	+	+	+	+	−	−	+	+	+	+	−	−
2	+	+	+	+	+	+	−	−	+	+	+	+	−	−
3	+	+	+	+	+	+	−	−	+	+	+	+	−	−
4	+	+	+	+	+	+	−	−	+	+	+	+	−	−
5	+	+	+	+	+	+	−	−	+	+	+	+	−	−
6	+	+	+	+	+	+	−	−	+	+	+	+	−	−
7	+	+	+	+	+	+	−	−	+	+	+	+	−	−
8	+	+	+	+	+	+	−	−	+	+	+	+	−	−
9	+	+	+	+	+	+	−	−	−	−	−	−	+	+
10	+	+	+	+	+	+	−	−	+	+	+	+	−	−
11[c]	+	+	+	+	+	+	−	−	+	+	+	+	−	−
12[d]	+	+	+	+	−	+	−	−	+	+	+	+	−	−
13	+	+	+	+	+	+	−	−	+	+	+	+	−	−
14	+	+	+	+	+	+	−	−	+	+	+	+	−	−
Control	+	+	+	+	+	+	−	−	+	+	+	+	−	−
	In Agreement with Control Laboratory[e]													
No.	12	13	13	13	13	13	13	13	12	12	12	12	12	12
%	92	100	100	100	100	100	100	100	92	92	92	92	92	92

[a] Number of *Salmonella* cells per level given in Table 1.
[b] Lactose broth modified by addition of 1% $NaHCO_3$ and brilliant green dye (final concentration 0.002%).
[c] Initiated analysis 1 day late.
[d] Results of Collaborator 12 were excluded from analysis; collaborator neglected to use both selective broths.
[e] Evaluation based on a total of 13 collaborators.

NFDM-BG and BPW as pre-enrichments. In no instance was *Salmonella* detected in a sample pre-enriched in BPW but not detected in the replicate sample pre-enriched in NFDM-BG broth.

The relative efficiency of the 2 pre-enrichment media for recovering *Salmonella* from 2 types of casein powder is shown in Table 3. For samples of lactic casein inoculated with *S. infantis* at levels 1–3 and samples of acid casein inoculated with *S. anatum* at levels 1–2, the control laboratory detected *Salmonella* in the 2 replicate samples of all levels pre-enriched by lactose broth or by modified lactose broth. Collaborator 11 initiated analysis 1 day late; however, this did not adversely affect results for lactic or acid casein since results for both products were in complete agreement with control laboratory results. Excluding the results of Collaborator 12, comparisons were made of the relative efficiency of 2 pre-enrichments for recovering *Salmonella* for 39 positive lactic casein samples and 13 negative lactic casein samples. *Salmonella* was detected in all 39 (100%) of the lactic casein samples inoculated with *S. infantis* and pre-enriched in modified lactose broth and in 38 of 39 (97.4%) of the replicate samples pre-enriched in lactose broth. No *Salmonella* were recovered from the uninoculated lactic casein samples. The relative efficiency of 2 pre-enrichment broths for detecting *Salmonella* was compared in 26 positive acid casein samples and 13 uninoculated acid casein samples. Results from 13 collaborators indicated that *S. anatum* was detected in 24 of 26 (92.3%) comparisons at both inocula levels in samples pre-enriched by lactose broth or by modified lactose broth. Conversely, 12 of 13 (92.3%) uninoculated samples were reported as *Salmonella*-negative. Analytical results of acid casein reported by 12 of 13 collaborators agreed. Since all discrepant results were reported by Collaborator 9, this participant apparently mishandled samples representing all 3 levels of acid casein.

Overall results from 13 collaborators in the analysis of milk chocolate indicated that 102 of 104 (98.1%) determinations for NFDM-BG and 100 of 104 (96.2%) determinations for BPW were in agreement with results from the control laboratory. Both media detected *Salmonella* from positive milk chocolate at the lowest level of contamination (≤ 1/g) employed in this study, with no statistical difference between recovery

results reported for either pre-enrichment broth. Even though the performance of NFDM-BG for the isolation of *Salmonella* was comparable to that of BPW, the following factors must be considered before one of these pre-enrichment broths is recommended for inclusion in AOAC methods: BPW is not available in dehydrated form and must be prepared from individual ingredients a week before use. If this preparation is not made with precision, the final medium may lack uniformity and adversely affect the level of performance in food analyses. In a comprehensive study of methods for the analysis of milk chocolate contaminated with *Salmonella*, Wilson et al. (1) demonstrated the superiority of NFDM-BG over BPW. Therefore, NFDM-BG should be used for analysis of milk chocolate contaminated with *Salmonella*.

Overall results from 13 collaborators in the analysis of casein indicated that of 91 determinations for lactose broth and modified lactose broth, 87 (95.6%) and 88 (96.7%), respectively, were in agreement with results from the control laboratory. Both broths detected *Salmonella* from casein at the lowest level of contamination (<1/g) used in this study, with no statistical difference between results reported for either pre-enrichment broth evaluated. Although the use of brilliant green dye as a selective ingredient to reduce the growth of competing non-*Salmonella* organisms is widely recognized, this dye has been shown to inhibit the growth of *Salmonella*. Because the microbiological counts of casein are usually low, the addition of brilliant green dye to lactose broth for the analysis of casein would seem to be unwarranted. The addition of $NaHCO_3$ to lactose broth results in an initial pH of 8.3, allows dissolution of casein, and makes pipetting to selective broths easier; however, casein lactose broth without $NaHCO_3$ can be subcultured with the large bore pipets commonly used in food analysis. In a comprehensive study of *Salmonella* serotypes, Poelma et al. (3) demonstrated that lactose broth gave more consistent recoveries of low levels of *Salmonella* than did modified lactose broth. For this reason, lactose broth should be used for pre-enrichment of edible casein.

It is recommended that the official final action method for the detection of *Salmonella*, **46.054–46.067**, be revised to include applicability to milk chocolate with pre-enrichment in reconstituted NFDM-BG (final concentration 0.002%) and applicability to edible casein with pre-enrichment in lactose broth (without modification).

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Comparative Efficiency of Brilliant Green, Bismuth Sulfite, *Salmonella-Shigella*, Hektoen Enteric, and Xylose Lysine Desoxycholate Agars for the Recovery of *Salmonella* from Foods: Collaborative Study

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The relative efficiency of brilliant green (BG), bismuth sulfite (BS), *Salmonella-Shigella* (SS), xylose lysine desoxycholate (XLD), and Hektoen enteric (HE) agars for the recovery of *Salmonella* from 5 foods was collaboratively studied in 11 laboratories. The analytical efficiency of various paired combinations of the 5 agars was statistically compared according to 3 parameters: (*1*) productivity or recovery of *Salmonella*, (*2*) rate of enumeration of cultures that were false positive for *Salmonella*, and (*3*) rate of enumeration of false-negative reactions. In descending order of productivity, the sequential ranking was BS, XLD, HE, BG, and SS agars. In ascending order, the rates of false-positive reactions based on a statistical analysis of paired agar combinations was HE, BS, BG and XLD (tie), and SS agars. Analogously, in ascending order, the sequence of false-negative reaction rates was BS, XLD, HE, BG, and SS agars. The combination of BS, XLD, and HE agars is more efficient for recovery of *Salmonella* from foods than is the present official combination of BG, BS, and SS agars. The revision of official final action method 46.054 to replace the combination of BG, BS, and SS agars with a combination of BS, XLD, and HE agars has been adopted official first action.

In the current method for *Salmonella* in foods, 3 selective agars are used to isolate pure *Salmonella*-suspicious colonies, the choice of brilliant green (BG), bismuth sulfite (BS), and *Salmonella-Shigella* (SS) agars being based on the 1967 report of the Associate Referee on *Salmonella* (1). Since then, we have frequently observed in our laboratory that the *Salmonella* productivity of the SS agar has not been comparable to that of the BG and BS agars. Moreover, considerable time has been wasted in picking *Salmonella*-suspicious colonies on this agar which could not be biochemically and serologically confirmed as *Salmonella*. Analysts using the AOAC method for isolating *Salmonella* (2) are required to pick all "uncolored to pale pink, opaque, transparent or translucent . . . colonies." This type of colonial morphology could be expected to appear from those organisms unable to utilize lactose, which changes the neutral red indicator dye in the SS agar to the acidic (red) color range. Like *Salmonella*, members of the genera *Proteus* and *Citrobacter* and certain *Enterobacter* species have negative, or, in some instances, variable lactose utilization reactions (3). Consequently, these organisms can be expected to closely resemble *Salmonella* colonies on the SS agar. The picking of these non-*Salmonella* colonies is largely responsible for the somewhat lower productivity of the SS agar relative to that of the BG and BS agars.

Since 1967, several other selective agars for the isolation of *Salmonella* have been advocated, including xylose lysine desoxycholate (XLD) agar introduced by Taylor (4) in 1965. Actually, XLD agar is one of a family of agars. Xylose lysine base (XL) agar, considered a nonselective medium, permits the growth of most enteric bacteria. When supplemented with sodium thiosulfate and ferric ammonium citrate, the basal medium becomes complete and, when further supplemented with either sodium desoxycholate or brilliant green dye, becomes XLD or xylose lysine brilliant green (XLBG) agar, respectively. Ironically, the XLD agar was originally designed by Taylor for the isolation of *Shigella* and *Providencia*, and XLBG for the isolation of *Salmonella*. We have used XLD for the isolation of *Salmonella* from foods, however.

Hektoen enteric (HE) agar, introduced by King

This report of the Associate Referee, P. L. Poelma, was presented at the 94th Annual Meeting of the AOAC, Oct. 20-23, 1980, at Washington, DC.

This recommendation was approved by the General Referee and by Committee F, and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1981) **64**, 408.

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and Metzger (5, 6) in 1968, is another selective agar recommended for the isolation of enteric pathogens. This agar was originally designed for the isolation of *Shigella* from clinical specimens but was found to be applicable for the isolation of *Salmonella*. HE agar differed from all other enteric selective agars in containing an indicator system consisting of bromthymol blue and Andrade's indicator. It also contains increased amounts of fermentable carbohydrates and peptones along with a selectively inhibitory system consisting of bile salts and sodium desoxycholate.

Recently, we reported (7) a comparative evaluation of BG, BS, SS, HE, and XLD agars for the recovery of *Salmonella* from foods in which these 5 selective agars were used on naturally contaminated food samples over an extended period. That study demonstrated the inadequacy of the SS agar and the advisability of using a 4-agar system (BG, BS, HE, and XLD) to recover a maximal number of *Salmonella* isolates. Based on the results of that evaluation, a collaborative study was undertaken of the comparative efficiency of these 5 agars for the isolation of *Salmonella* from selected food types.

Collaborative Study

Twelve laboratories participated in this collaborative study. Results from one laboratory, however, were not included because of inadequate biochemical and serological confirmation of *Salmonella* cultures. Each collaborator was furnished with 1 lb each of BG, BS, SS, HE, and XLD agars which had been screened and found acceptable by the originating laboratory (Division of Microbiology) in plating efficiency tests. All collaborators were supplied with the same lot of BG, BS, SS, and HE agars; 2 collaborators were supplied with a lot number of XLD agar different from that used by the other 9 collaborators. Collaborators were asked to prepare the BG, BS, and SS agars according to AOAC specifications (2) and to prepare the HE and XLD agars according to the manufacturer's instructions. Collaborators were asked to prepare the BS agar plates the day before they were streaked, based on the report that freshly prepared batches of BS agar were extremely toxic to *S. typhimurium* (8), and to prepare plates of the other 4 agars on the day they were to be streaked. After completion of the study, however, it was learned that some collaborators prepared plates of certain selective agars, other than the BS agar, the day before the agars were streaked. Moreover, because of the large number of selective agar plates required to determine levels of *Salmonella* in the samples, the originating laboratory had to make these particular selective agar plates the day before the plates were streaked.

Each laboratory was furnished with 10 samples of each of 5 foods: milk chocolate, soy protein powder, sunflower seeds, brown rice, and soy flour. Milk chocolate was used because of its involvement in several documented cases of salmonellosis (9); selection of the other 4 foods was based on the occurrence of *Salmonella* in these types of foods reported in a recent bacteriological survey of health foods (10). The 5 foods were obtained either in bulk or in available retail-sized packages from local retail outlets. The packages were pooled, mixed manually, and artificially inoculated with varying levels and combinations of *Salmonella*, *Escherichia*, *Enterobacter*, *Proteus*, and *Citrobacter* microorganisms. Samples were inoculated with cultures preselected from our stock culture collection by screening to demonstrate shortcomings of the agars as experienced in our laboratory or as reported in the literature. One example was the inclusion of lactose-positive *Salmonella* cultures which would not ordinarily appear as typical *Salmonella* colonies on plates of BG and SS agars. The inoculum cultures were grown overnight at 35°C in brain heart infusion or lactose broth, centrifuged (3015 × *g*), and washed twice in Butterfield's phosphate buffer (pH 6.8–7.2). In selected instances with the samples of soy flour, the washed cell suspensions were heat-shocked 10 min in a 55°C water bath before inoculation into the food samples. One sample in each series of 10 samples served as the uninoculated negative control. Other controls included positive and negative media controls. A jar of uninoculated pre-enrichment broth treated like the collaborative study samples served as the negative media control. Each collaborator was furnished with an H_2S-positive and an H_2S-negative *Salmonella* culture to be used as positive media controls in the analytical protocol. Analyses were scheduled so that a series of 10 samples of a particular food was examined by each collaborator every 14–15 days.

The milk chocolate samples were inoculated as follows: 700 g (approximate) portions of milk chocolate were melted either in a double boiler or in an Arnold steamer and then allowed to equilibrate in a water bath at 59–60°C. The molten chocolate mass was transferred to a mixing bowl, and different levels of pooled *Salmonella* serotypes and pooled *E. coli* strains were added to the chocolate. The artificially inocu-

lated chocolate was mixed by an electric mixer. The homogenized chocolate was then spread onto plastic sheets and allowed to solidify. Afterwards, 30 g (approximate) portions were weighed into sterile plastic bags, coded with a randomized, singular 5-digit number, and sent to participating laboratories. All samples were inoculated 14–15 days before collaborators began their analyses to give microorganisms sufficient time to stabilize within the sample. Moreover, this 2-week storage period, during which time the inoculated cells received no nourishment, could in itself be considered a type of stress.

The samples of soy protein powder, sunflower seeds, brown rice, and soy flour were all inoculated in the same manner. Before inoculation of the sunflower seeds, however, 400 g portions of some collaborative samples of these seeds were autoclaved 30 min at 121°C and dried in a forced-air drying oven 24 h at 68°C to ensure that none of the indigenous microflora would interfere in the enumeration of inoculated *Proteus* and *Citrobacter* microorganisms. Even though selected samples of brown rice were also inoculated with *Proteus* and *Citrobacter*, these samples were not autoclaved before inoculation. From the bulk volumes of each of the foods, 30 g portions were weighed into sterile plastic bags and inoculated with different levels and combinations of *Salmonella* and non-*Salmonella* microorganisms. In some instances single serotypes or strains of *Salmonella*, *E. coli*, *Enterobacter*, *Citrobacter*, and *Proteus* were used; in other instances pools of 2 or more serotypes or strains of these genera were used. The inoculated samples were mixed manually and then number-coded. For each of the 5 selective agars used in this study, the Division of Microbiology determined most probable number (MPN) values of microorganisms 1 day and 14–15 days after inoculation (14–15 days was the time collaborators began analyses).

Collaborators were instructed as follows:

From each 30–32 g (30 g dry weight plus weight of inocula) collaborative sample, weigh 25 g. Pre-enrich samples of milk chocolate in reconstituted nonfat dry milk with added brilliant green dye (final concentration, 0.02 mg dye/mL) or crystal violet dye (final concentration, 0.04 mg dye/mL); pre-enrich all other samples in lactose broth. Maintain a sample/pre-enrichment broth ratio of 1:9 for all analyses. Blend sample/broth mixtures 2 min, let stand 60 min, and then adjust to pH 6.8 ± 0.2. Incubate pre-enriched collaborative samples 24 ± 2 h at 35°C; then subculture 1 mL aliquots to 10 mL selenite cystine (SC) broth or 10 mL tetrathionate (TT) broth. Incubate these selective enrichments 24 ± 2 h at 35°C and streak to each of the 5 selective agars. Incubate the selective agar plates at 35°C and examine at 24 ± 2 h. If the BS plates do not have growth or suspicious *Salmonella* colonies, incubate them an additional 24 ± 2 h. Pick typical *Salmonella* colonies to triple sugar iron agar and lysine iron agar; confirm biochemically and group somatically where possible.

In determining *Salmonella* MPN values of organisms inoculated into collaborative samples, chocolate samples were blended with nonfat dry milk containing added BG dye (final concentration, 0.02 mg dye/mL), whereas samples of the other 4 food types were blended with lactose broth. The originating laboratory had to make a few concessions in the analytical protocol, however, because of the extraordinarily large number of tubes and plates to be analyzed. First, no attempt was made to adjust the pH of each individual tube of pre-enrichment broth used in the MPN determination. Second, the originating laboratory prepared SC broth by heating rehydrated medium to boiling and dispensing 10 mL portions into nonsterile test tubes. Tubed medium was not heated in flowing steam. Moreover, it was logistically necessary to make both SC and basal TT broth 1–4 days before use and store it under refrigeration. Third, since we have found that most *Salmonella* cultures produce H_2S on BS, HE, and XLD agars, greater emphasis was placed on picking H_2S-positive, rather than H_2S-negative, colonies on these particular agars. The collaborating analysts, however, were not instructed to pick only H_2S-positive colonies. Next, the highest dilution of sample which gave suspicious colonies on all 3 plates (3-tube MPN) of any particular selective agar and, in general, the next 2 succeeding higher dilutions were confirmed in calculating MPN values. No attempt was made to confirm cultures in all lower dilutions. "Skips" occurring in lower dilutions could be attributable to overgrowth by competing organisms. Finally, it was not always possible to incubate exactly 24 ± 2 h for pre-enrichments, selective enrichments, and selective agars.

Certain samples were inoculated with non-*Salmonella* bacteria, which in most instances served as competing microflora to the *Salmonella* bacteria also present. In the case of sunflower seeds, however, each collaborator was sent 10 samples, 2 of which contained either *Citrobacter* or *Proteus* only. Since the SC and TT broths used in the enumeration of *Salmonella* are intended to inhibit competing non-*Salmonella* microorgan-

isms, higher MPN values of *Citrobacter* and *Proteus* could be expected if the selective enrichment step were bypassed. Accordingly, MPN levels of *Citrobacter* and *Proteus* were determined by blending food samples with lactose broth, diluting, and inoculating into tubes of lactose broth. After overnight incubation at 35°C, the tubes were streaked directly to the selective agars. Isolates were picked and identified biochemically. In enumerating *E. coli* and *Enterobacter*, samples were blended with Butterfield's phosphate buffer and serial dilutions were inoculated into tubes of lauryl (sulfate) tryptose (LST) broth. Tubes of LST broth were examined for gas formation at 24 and 48 ± 2 h. In enumerating *E. coli*, the following deviations were made from the official AOAC method. First, one or two 3 mm loopfuls of broth from gassing LST tubes were transferred to EC medium and incubated in a water bath at 45.5 ± 0.3°C, rather than at 45.5 ± 0.05°C. Secondly, the tubes of EC medium were inadvertently submerged in the water bath so that the water level was above the highest level of medium in the main tube rather than above the highest level of medium in the fermentation vial. Moreover, 10 mL portions of EC medium were dispensed in 20 × 150 mm tubes, rather than 8 mL portions in 16 × 150 mm tubes. EC tubes gassing at 24 or 48 ± 2 h were streaked to Levine EMB agar. Colonies likely to be *E. coli* were picked to LST broth, rather than plate count agar slants, and were incubated at 35°C and examined at 24 and 48 ± 2 h intervals. A small amount of broth was transferred with a sterile needle from gassing LST tubes to tubes of Koser citrate, 1% tryptone, and MRVP medium. Incubation of IMViC (indole, methyl red, Voges-Proskauer, and citrate utilization) media and performance of biochemical tests were in general accordance with AOAC recommendations (2), except creatine was not used to accelerate the test for the presence of acetylmethylcarbinol.

In enumerating *Enterobacter*, gassing LST tubes were streaked directly to Levine EMB agar and colonies were identified as described above. Cultures were classified as *E. coli* or *Enterobacter* based on the biochemical pattern of IMViC reactions.

A statistical pair-wise comparison of the recovery of *Salmonella* and of the rate of false-negative reactions for any particular food was performed as follows: The proportion of samples determined to contain *Salmonella* was expressed as $P_i = r/n$, where r is the total number of samples that contained *Salmonella* for a given selective enrichment/agar combination and n is the total number of positive samples. To compare the results for 2 given enrichment/agar combinations, P_i and P_j, the following formula was used:

$$Z = (P_i - P_j)/S_{P_i - P_j}$$

where $S_{P_i - P_j}$ is the standard error of the difference between P_i and P_j and is computed as

$$S_{P_i - P_j} = [(b + c - (b - c)^2/n)/n(n - 1)]^{1/2}$$

where b and c are the numbers of samples for which the 2 selective enrichment/agar combinations disagreed in terms of the presence of *Salmonella* and n is the total number of samples.

A statistical pair-wise comparison of the rate of false-positive reactions was performed as follows: The proportion of false-positive cultures for each selective enrichment/agar combination was computed as

$$R = \sum y_i / \sum x_i$$

where Σy_i is the total number of false-positive cultures obtained by all collaborators for any particular food type and Σx_i is the total number of cultures picked that could be identified as *Salmonella* or non-*Salmonella* by all collaborators for any particular food type. Since y and x varied from collaborator to collaborator, the standard error of R was computed as:

$$S_R = 1/(n\bar{x})^{1/2}\,[(\sum y_i^2 - 2R\sum y_i x_i + R^2\sum x_i^2)/n - 1]^{1/2}$$

where n is the total number of collaborating laboratories and $\bar{x} = \Sigma x_i/n$. To compare the results for any 2 given selective enrichment/agar combinations, the following formula was used:

$$Z = (R_i - R_j)/[(S_{R_i})^2 + (S_{R_j})^2]^{1/2}.$$

An absolute value of $Z > 1.96$ was taken to indicate that the 2 ratios were significantly different at the 5% probability level.

Results and Recommendations

Table 1 shows the level of microorganisms in the samples of milk chocolate determined by the originating laboratory 1 day and 14 days after inoculation. Samples 1–9 contained various dilutions of a pool consisting of *S. paratyphi B* and *S. poona*. Although *E. coli* was not inoculated into samples 1–3, samples 4–9 contained various dilutions of a pool consisting of 5 strains of *E. coli*. We inoculated these samples in order to have low levels of *Salmonella* present in the samples at the time the collaborators began analyses. The tenth

Table 1. Enumeration of microorganisms in artificially contaminated milk chocolate samples

Sample No.	Salmonella Serotype(s)	Salmonella Dilution[a]	Salmonella MPN/g: Selenite cystine broth BG[b]	Selenite cystine broth BS[c]	Selenite cystine broth SS[d]	Selenite cystine broth HE[e]	Selenite cystine broth XLD[f]	Tetrathionate broth BG	Tetrathionate broth BS	Tetrathionate broth SS	Tetrathionate broth HE	Tetrathionate broth XLD	Escherichia coli[g] Dilution[a]	Escherichia coli[g] MPN/g
1	S. paratyphi B, S. poona	10^0	93[h] (0.09)	93 (0.09)	93 (0.09)	93 (0.09)	93 (0.09)	93 (0.09)	93 (0.09)	93 (0.09)	93 (0.09)	93 (0.09)	-	< 0.3 (< 0.3)
2	S. paratyphi B, S. poona	10^{-1}	9.3 (0.04)	9.3 (0.04)	9.3 (0.04)	9.3 (0.04)	9.3 (0.04)	9.3 (0.04)	9.3 (0.04)	9.3 (0.04)	9.3 (0.04)	9.3 (0.04)	-	< 0.3 (< 0.3)
3	S. paratyphi B, S. poona	10^{-2}	1.5 (< 0.03)	1.5 (< 0.03)	1.5 (< 0.03)	1.5 (< 0.03)	1.5 (< 0.03)	1.5 (< 0.03)	1.5 (< 0.03)	1.5 (< 0.03)	1.5 (< 0.03)	1.5 (< 0.03)	-	< 0.3 (< 0.3)
4	S. paratyphi B, S. poona	10^0	23 (0.03)	23 (0.03)	23 (0.03)	23 (0.03)	23 (0.03)	23 (0.03)	23 (0.03)	23 (0.03)	23 (0.03)	23 (0.03)	10^0	9.3 (< 0.3)
5	S. paratyphi B, S. poona	10^{-1}	4.3 (0.04)	0.15 (0.04)	4.3 (0.04)	4.3 (0.04)	4.3 (0.04)	23 (0.04)	9.3 (0.04)	23 (0.04)	23 (0.04)	9.3 (0.04)	10^0	4.3 (< 0.3)
6	S. paratyphi B, S. poona	10^{-1}	9.3 (< 0.03)	9.3 (< 0.03)	9.3 (< 0.03)	9.3 (< 0.03)	9.3 (< 0.03)	9.3 (< 0.03)	9.3 (< 0.03)	9.3 (< 0.03)	9.3 (< 0.03)	9.3 (< 0.03)	10^{-1}	0.91 (< 0.3)
7	S. paratyphi B, S. poona	10^{-2}	0.03 (< 0.03)	0.03 (< 0.03)	0.43 (< 0.03)	0.07 (< 0.03)	0.07 (< 0.03)	0.43 (< 0.03)	0.43 (< 0.03)	0.43 (< 0.03)	0.43 (< 0.03)	0.43 (< 0.03)	10^0	2.3 (< 0.3)
8	S. paratyphi B, S. poona	10^{-2}	0.04 (< 0.03)	0.04 (< 0.03)	0.09 (< 0.03)	0.04 (< 0.03)	0.04 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	10^{-1}	< 0.3 (< 0.3)
9	S. paratyphi B, S. poona	10^{-2}	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	0.23 (< 0.03)	10^{-2}	< 0.3 (< 0.3)
10	None	-	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	-	< 0.3 (< 0.3)

[a] One mL of indicated dilution was inoculated into 700 g amounts of chocolate.

[b-f] BG, brilliant green; BS, bismuth sulfite; SS, Salmonella-Shigella; HE, Hektoen enteric; XLD, xylose lysine desoxycholate agars.

[g] Pool consisting of 5 strains of E. coli.

[h] Numbers not in parentheses are MPN values of microorganisms in spiked samples 1 day after inoculation. Numbers in parentheses are MPN values 14 days after inoculation, when actual collaborative study sample analyses were begun.

Table 2. Recovery of *Salmonella* from milk chocolate samples using 5 selective agars [a]

		Selenite cystine					Tetrathionate				
Coll.	Sample No.	BG	BS	SS	HE	XLD	BG	BS	SS	HE	XLD
1	1	+[b]	+	+	+	+	+	+	+	+	+
	2	+	+	-	+	+	+	+	-	+	+
	3	-	-	-	-	-	-	-	-	-	-
	4	-	+	+	+	+	+	+	+	+	+
	5	-	-	-	-	-	-	-	-	-	-
	6	+	+	+	+	+	+	+	+	+	+
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
2	1	+	+	-	+	+	+	+	+	+	+
	2	-	-	-	-	-	-	-	-	-	-
	3	+	-	-	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
3[c]	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	-	-	-	-	-	-	-	-	-	-
	4	+	-	+	+	-	+	+	+	+	+
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
4	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	-	+	+
	3	-	-	-	-	-	-	-	-	-	-
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
5	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	-	-	-	-	-	-	-	-	-	-
	4	+	+	+	+	+	+	+	+	+	+
	5	-	-	-	-	-	-	-	-	-	-
	6	+	+	+	+	+	+	+	+	+	+
	7	-	-	-	-	-	-	-	-	-	-
	8	+	+	+	+	+	+	+	+	+	+
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
6	1	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-
	4	+	+	+	+	+	+	+	+	+	-
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
7	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	-	-	-	-	-	-	-	-	-	-
	4	+	+	+	+	+	+	+	+	+	+
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-

Table 2. (cont'd)

Coll.	Sample No.	Selenite cystine BG	BS	SS	HE	XLD	Tetrathionate BG	BS	SS	HE	XLD
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
8[d]	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	-	-	-	-	-	-	-	-	-	-
	4	+	+	+	+	-	+	+	+	+	+
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
9	1	+	+	-	-	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	-	-	-	-	-	-	-	-	-	-
	4	+	+	+	+	+	+	+	+	+	+
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
10	1	+	+	-	+	+	+	+	+	+	+
	2	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	7	+	+	-	-	+	+	+	-	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
11	1	-	-	+	-	-	-	-	+	-	-
	2	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-
	5	+	+	+	+	+	+	+	+	+	+
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
	Totals	33 (94)[e]	32 (91)	29 (83)	32 (91)	32 (91)	34 (97)	34 (97)	32 (91)	34 (97)	33 (94)

[a] For identification of agars, see Table 1, footnotes b–f.

[b] +, Salmonella recovered; -, Salmonella not recovered.

[c] Seven of 10 samples were pre-enriched in reconstituted nonfat dry milk with 10 times the recommended concentration of brilliant green dye.

[d] Collaborator initiated samples 3 days late.

[e] Percentage of total number of 35 positive samples.

sample of milk chocolate and of the other 4 foods was not inoculated with any microorganisms and served as the negative control. All 9 of the *Salmonella*-inoculated samples contained viable *Salmonella* microorganisms 1 day after inoculation but only 4 of these samples (1, 2, 4, 5) contained determinant levels of *Salmonella* 14 days after incubation as determined by the originating laboratory.

The recovery of *Salmonella* from the milk chocolate samples by the collaborators and originating laboratory is shown in Table 2. Thirty-five samples found positive by the various laboratories were distributed as follows: 5 samples (2 labs), 4 samples (1 lab.), 3 samples (6 labs), 2 samples (1 lab.), and 1 sample (1 lab.). Even though we attempted to thoroughly homogenize the inoculated *Salmonella* micro-

organisms in the molten milk chocolate, it is evident that the *Salmonella* microorganisms were present in low numbers, and even then, only intermittently. Before inoculation with *Salmonella*, the milk chocolate was melted and allowed to equilibrate in a water bath at 59–60°C, which could be expected to substantially reduce the level of *Salmonella* organisms and to injure the surviving cell population. Nevertheless, the relative performance of the 5 selective agars for isolating *Salmonella*, which was our main concern, could still be determined. All 5 agars were higher in *Salmonella* productivity when streaked from TT broth rather than from SC broth, productivity being defined as the number of *Salmonella*-positive samples obtained by using any one selective enrichment/selective agar combination divided by the total number of *Salmonella*-positive samples obtained by using all 10 selective enrichment/selective agar combinations. No one selective enrichment/selective agar combination was able to recover 100% of the *Salmonella*-positive samples. However, the TT–BG, TT–BS, TT–HE combinations were able to recover 34 (97%) of the 35 positive samples.

The enumeration of microorganisms inoculated into samples of soy protein powder is shown in Table 3. Samples 1 and 2 contained *S. anatum* as the single serotype; no *E. coli* was inoculated into these samples. This particular strain of *S. anatum* was lactose-positive. Throughout the study it was emphasized that only colonies that were typical in appearance for *Salmonella* should have been picked from the plates of selective agars. It should be readily realized, however, that in the analysis of food samples in a strictly official regulatory capacity, both atypical and typical colonies would be picked in an FDA laboratory. The intent of this study was not to determine if all 5 agars would be able to detect *Salmonella* in any given sample, regardless of the morphological appearance of the various colony types on the selective agars. Rather, we were concerned with the relative efficiency of these 5 agars in discriminating *Salmonella* from the competing non-*Salmonella* microorganisms. Since one is more inclined to pick typical, rather than nontypical, colonies in any given situation, the greater the number of times a *Salmonella* culture appears as a typical colony on any one agar, the more apt that agar will be to detect any *Salmonella* organisms that may be present. Moreover, picking an excessive number of atypical colonies in this study would have led to a large number of false-positive reactions which would have distorted the efficiency of the various agars. Samples 3–9 were inoculated with various dilutions of a pool of 2 *Salmonella* serotypes, *S. derby* and *S. virchow*. Samples 3, 4, 6, 7, 8 were each inoculated with different dilutions of a pool consisting of 5 strains of *E. coli*. For the most part, levels of *Salmonella* at the time of initiation of analyses of the soy protein powder samples were higher than those levels present in the chocolate collaborative samples, particularly for samples 3, 4, and 5 of the soy protein powder. It can be stated that all 9 of the *Salmonella*-inoculated samples contained viable *Salmonella* cells at the time of initiation of sample analyses if the following 2 assumptions are made: First, that *Salmonella* microorganisms were uniformly distributed within the 30–32 g collaborator portions from which a 25 g analytical unit was taken for each sample number; second, that all the cells did not die in any one sample sent to a particular laboratory. This second assumption is somewhat tenuous, however, and should be kept in mind when interpreting the results from any one collaborator.

Table 4 shows the *Salmonella* recovery patterns obtained by the collaborators for the samples of soy protein powder. Since BG and SS agars both contain lactose, the appearance of the lactose-positive strain of *S. anatum* would have been atypical, and for the criteria established for this study, would not have been expected to be detected on these 2 agars for the first 2 samples. Of the 3 *Salmonella* selective agars recommended by the AOAC (2) and the *Bacteriological Analytical Manual* (BAM) (11), the lactose-positive strain of *S. anatum* should have appeared typical only on the BS agar. For these first 2 samples, collaborators obtained different results with the HE and XLD agars. Samples 3–9 were inoculated with different dilutions of a pool of *S. derby* and *S. virchow*, both of which were H_2S-positive. All collaborators except Collaborator 1 reported that the SS agar was less productive than the other 4 agars when streaked from SC broth, TT broth, or both. Similarly, the overall combined results of all laboratories demonstrated that the SS agar was less productive than the other 4 agars when streaked from both selective enrichments. The SC–SS and TT–SS selective enrichment/selective agar combinations were able to recover only 52 and 44%, respectively, of the *Salmonella*-positive samples, somewhat less than recoveries obtained with the other 4 agars. The selective enrichment/selective agar combination giving the highest recoveries of *Salmonella* were SC–BS, TT–BS, TT–HE, and TT–XLD.

Table 3. Enumeration of microorganisms in artificially contaminated soy protein powder samples[a]

Sample No.	Salmonella Serotype(s)	Dilution[b]	Salmonella MPN/g: Selenite cystine broth BG	BS	SS	HE	XLD	Tetrathionate broth BG	BS	SS	HE	XLD	Escherichia coli[c] Dilution[b]	Escherichia coli MPN/g
1	S. anatum	10^{-3}	< 0.03[d] (< 0.03)	93 (2.3)	< 0.03 (< 0.03)	0.11 (0.15)	93 (2.3)	< 0.03 (< 0.03)	93 (2.3)	< 0.03 (< 0.03)	0.15 (0.11)	93 (2.3)	-	< 0.3 (< 0.3)
2	S. anatum	10^{-4}	< 0.03 (< 0.03)	2.3 (0.93)	< 0.03 (< 0.03)	0.09 (0.04)	2.3 (0.15)	< 0.03 (< 0.03)	2.3 (0.93)	< 0.03 (< 0.03)	0.09 (0.07)	2.3 (0.93)	-	< 0.3 (< 0.3)
3	S. derby, S. virchow	10^{-2}	≥9,300 (≥23,000)	≥9,300 (≥23,000)	≥2,100 (≥23,000)	≥9,300 (≥23,000)	≥9,300 (≥23,000)	≥9,300 (≥23,000)	≥9,300 (≥23,000)	ND (≥23,000)	≥9,300 (≥23,000)	≥9,300 (≥23,000)	10^{-1}	15,000 (23,000)
4	S. derby, S. virchow	10^{-2}	≥43,000 (9,300)	≥43,000 (9,300)	≥43,000 (9,300)	≥43,000 (9,300)	≥43,000 (9,300)	≥43,000 (9,300)	≥43,000 (9,300)	≥43,000 (9,300)	≥43,000 (9,300)	≥43,000 (9,300)	10^{-2}	9,300 930
5	S. derby, S. virchow	10^{-2}	≥ 23,000 (≥21,000)	≥23,000 (≥21,000)	≥ 23,000 ND[e]	≥23,000 (≥21,000)	≥23,000 (≥21,000)	≥ 23,000 (≥21,000)	≥ 23,000 (≥21,000)	≥ 23,000 ND	≥ 23,000 (≥21,000)	≥23,000 (≥21,000)	-	< 0.3 (< 0.3)
6	S. derby, S. virchow	10^{-4}	43 (93)	43 (93)	0.06 (43)	23 (93)	43 (43)	43 (93)	43 (93)	0.04 (43)	23 (43)	43 (43)	10^{-2}	≥2,300,000 (2,300)
7	S. derby, S. virchow	10^{-4}	930 (23)	930 (23)	930 (23)	930 (23)	930 (23)	930 (23)	930 (23)	930 (23)	930 (23)	930 (23)	10^{-3}	4,300 (9.3)
8	S. derby, S. virchow	10^{-4}	43 (23)	43 (23)	43 (23)	43 (23)	43 (23)	43 (23)	43 (23)	0.2 (23)	43 (23)	43 (23)	10^{-4}	9.3 (43)
9	S. derby, S. virchow	10^{-4}	43 (93)	43 (93)	43 (93)	43 (93)	43 (93)	43 (93)	43 (93)	43 (93)	43 (93)	43 (93)	-	< 0.3 (< 0.3)
10	None	-	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	-	< 0.3 (< 0.3)

[a] For identification of agars, see Table 1, footnotes b-f.

[b] One mL of indicated dilution was inoculated into 30 g samples.

[c] Pool consisting of 5 strains of E. coli.

[d] Numbers not in parentheses are MPN values of microorganisms in spiked samples 1 day after inoculation. Numbers in parentheses are MPN values 14 days after inoculation, when actual collaborative study sample analyses were begun.

[e] Positive tube combination too improbable for MPN determination.

Table 4. Recovery of *Salmonella* from soy protein powder samples using 5 selective agars [a]

		Selenite cystine					Tetrathionate				
Coll.	Sample No.	BG	BS	SS	HE	XLD	BG	BS	SS	HE	XLD
1	1	-[b]	-	-	+	-	-	+	-	-	-
	2	-	-	-	-	-	-	+	+	+	+
	3	+	+	+	+	+	+	+	-	+	+
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
2	1	-	+	-	-	-	-	+	-	-	-
	2	-	+	-	-	-	-	+	-	-	-
	3	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	-	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	-	+	+	+	+	-	+	+
	7	+	+	+	+	+	+	+	-	+	+
	8	+	+	-	+	+	+	+	-	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
3	1	+	+	+	+	+	+	+	-	+	+
	2	-	+	-	+	-	-	+	-	-	+
	3	+	+	+	+	+	+	+	-	+	+
	4	+	+	-	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	-	+	+	+	+	+	+	+
	7	+	+	-	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
4	1	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-
	3	+	-	-	+	+	+	-	-	+	+
	4	+	+	-	+	+	+	+	-	+	+
	5	+	-	+	+	+	+	-	+	+	+
	6	+	-	-	+	+	+	-	-	+	+
	7	+	+	-	-	-	-	-	-	+	+
	8	+	-	-	+	+	+	-	-	+	+
	9	+	-	+	+	+	+	-	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
5	1	-	+	-	+	-	-	+	-	-	-
	2	-	+	-	-	+	-	+	-	-	+
	3	+	+	-	+	+	+	+	-	+	+
	4	+	+	-	+	+	+	+	-	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	-	-	-	+	+	-	+	+
	7	+	+	-	-	+	+	+	-	+	+
	8	+	+	-	+	+	+	+	-	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
6	1	-	+	-	-	+	-	+	-	+	+
	2	-	+	-	-	+	-	+	-	-	+
	3	+	+	-	+	+	+	+	-	+	+
	4	+	+	-	+	+	+	+	-	+	+
	5	+	+	-	+	+	+	+	-	+	+
	6	+	+	-	+	+	+	+	-	+	+
	7	+	+	-	+	+	+	+	-	+	+
	8	+	+	-	+	+	+	+	-	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
7	1	-	+	-	+	+	-	+	-	+	+
	2	-	+	-	-	+	-	+	-	+	+
	3	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	-	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	-	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-

Table 4. (cont'd)

Coll.	Sample No.	Selenite cystine BG	BS	SS	HE	XLD	Tetrathionate BG	BS	SS	HE	XLD
8	1	-	-	-	+	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	+	-
	3	+	+	-	+	+	+	+	-	+	+
	4	+	+	-	+	+	+	+	-	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	-	-	-	+	+	-	+	+
	7	+	+	-	+	+	+	+	-	+	+
	8	+	+	-	+	+	+	+	-	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
9	1	-	-	-	+	-	-	-	-	+	+
	2	-	-	-	-	-	-	-	-	-	-
	3	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	-	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	-	+	+
	7	+	+	+	+	+	+	+	-	+	+
	8	+	+	+	+	+	+	+	-	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
10	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	-	+	+
	4	+	+	+	+	+	+	+	-	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	-	+	+
	7	+	+	+	+	+	+	+	-	+	+
	8	+	+	+	+	+	+	+	-	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
11	1	-	+	-	-	-	-	+	-	-	-
	2	-	+	-	-	-	-	+	-	-	-
	3	+	+	+	+	+	+	+	+	-	+
	4	+	-	-	+	-	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	-	+	-	+	+	+	+	+
	7	+	+	-	+	+	+	+	+	+	+
	8	+	+	-	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
	Totals	80 (83)[c]	85 (89)	50 (52)	82 (85	80 (83)	79 (82)	87 (91)	42 (44	85 (89)	88 (92)

[a] For identification of agars, see Table 1, footnotes b-f.

[b] +, Salmonella recovered; -, Salmonella not recovered.

[c] Percentage of total number of 96 positive samples.

The levels of inoculated microorganisms in samples of sunflower seeds are shown in Table 5. Samples 1, 2, and 3 were each inoculated with the same level of a *Salmonella* pool consisting of 3 serotypes (*S. typhimurium*, *S. oranienburg*, and *S. enteriditis*) and each of these samples was challenged with different 10-fold dilutions of a pool consisting of 2 strains of *E. coli*. Yet, none of the identified colonies recovered was *S. enteriditis*. After the samples had been inoculated, the purity of the inocula cultures was determined by streaking onto each of the selective agars. It was subsequently discovered that one of the *E. coli* strains had been inadvertently contaminated with a culture that produced large mucoid-like colonies on certain of the selective agars This same culture was part of a pool used to inoculate samples of soy protein powder and brown rice. In the final analysis, however, this was not a determining factor since the levels of *E. coli* were established by determining the complete pattern of IMViC reactions. Samples 4-7 were inoculated with the same level of pool consisting of *S. derby* and *S. senftenberg*. Three of these samples (4-6) were each challenged with 10-fold dilutions of the *E. coli* pool as described above, whereas the *Salmonella* inoculum in sample 7 was not challenged with *E. coli*. Samples 8 and 9

Table 5. Enumeration of microorganisms in artificially contaminated sunflower seed samples [a]

Sample No.	Organism(s)	Dilution [b]	Salmonella, Citrobacter, and Proteus MPN/g										Escherichia coli [c]	
			Selenite cystine broth					Tetrathionate broth						
			BG	BS	SS	HE	XLD	BG	RS	SS	HE	XLD	Dilution [b]	MPN/g
1	S. typhimurium, S. oranienburg, S. enteriditis	10^{-4}	ND [d,e,f] (9.3)	23 (9.3)	ND (9.3)	ND (9.3)	ND (0.27)	ND (9.3)	23 (9.3)	ND (9.3)	ND (9.3)	ND 9.3	10^{-1}	≥ 930,000 (430)
2	S. typhimurium, S. oranienburg, S. enteriditis	10^{-4}	430 (0.43)	430 (0.43)	430 (0.07)	430 (0.43)	430 (0.43)	430 (0.43)	430 (0.43)	430 (0.43)	430 (0.43)	430 (0.43)	10^{-2}	7,500 (9,300)
3	S. typhimurium, S. oranienburg, S. enteriditis	10^{-4}	93 (0.93)	93 (0.93)	93 (0.23)	93 (0.93)	43 (0.93)	93 (0.93)	93 (0.93)	93 (0.93)	93 (0.93)	93 (0.93)	10^{-3}	430 (≥ 430)
4	S. derby, S. senftenberg	10^{-3}	1,500 (≥3,900)	1,500 (≥3,900)	1,500 (≥3,900)	1,500 (≥3,900)	1,500 (≥3,900)	1,500 (≥3,900)	1,500 (≥3,900)	1,500 (≥3,900)	1,500 (≥3,900)	1,500 (≥3,900)	10^{-1}	23,000 (4,300)
5	S. derby, S. senftenberg	10^{-3}	1,500 (230)	750 (230)	1,500 (230)	1,500 (230)	1,500 (230)	1,500 (230)	1,500 (230)	1,500 (230)	1,500 (230)	1,500 (43)	10^{-2}	93,000 (23,000)
6	S. derby, S. senftenberg	10^{-3}	430 (≥9,300)	430 (≥23,000)	430 (≥4,300)	430 (≥9,300)	430 (≥4,300)	430 (≥9,300)	430 (≥9,300)	430 (≥9,300)	430 (≥9,300)	430 (≥9,300)	10^{-3}	4,300 (430)
7	S. derby, S. senftenberg	10^{-3}	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	-	< 0.3 (< 0.3)
8	Citrobacter [g]	10^{-2}	≥ 3,900 (430)	≥ 3,900 (430)	≥ 3,900 (430)	≥ 3,900 (430)	≥ 3,900 (430)						-	< 0.3 (0.3)
9	Proteus [g]	10^{-2}	230 (0.11)	≥ 9,300 (230)	≥ 9,300 (230)	≥ 9,300 (230)	≥ 9,300 (230)						-	< 0.3 (< 0.3)
10	None	-	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	-	< 0.3 (< 0.3)

[a] For identification of agars, see Table 1, footnotes b-f.

[b] One mL of indicated dilution was inoculated into 30 g samples.

[c] Pool consisting of 2 strains of E. coli.

[d] Numbers not in parentheses are MPN values of microorganisms in spiked samples 1 day after inoculation. Numbers in parentheses are MPN values 14 days after inoculation, when actual collaborative study sample analyses were begun.

[e] Positive tube combination too improbable for MPN determination.

[f] In samples 1-3 examined 1 day after spiking, initiating laboratory found that some isolates agglutinated in E_4 somatic sera. No collaborators, however, reported E_4 isolates in collaborative study samples 1-3.

[g] All MPN values for Citrobacter and Proteus were obtained by streaking directly from lactose broth pre-enrichments.

were inoculated with the 10^{-2} dilution of a washed culture of *Citrobacter* or *Proteus*, respectively; sample 10 was the uninoculated negative control. Enumeration of *Proteus* was somewhat difficult because of its swarming ability and, in several instances, it was not always possible to pick *Proteus* colonies in pure culture in determining the MPN levels of this organism. A comparison of MPN values of *Proteus* as given by the 5 selective agars, however, demonstrates that the BG agar was particularly effective in suppressing this strain of *Proteus*, relative to the other 4 agars.

The recovery of *Salmonella* from the samples of sunflower seeds is shown in Table 6. A majority of the collaborators reported finding *Salmonella* in samples 1–7. The results from sample 2 were of particular interest since the ratio of *E. coli* to *Salmonella* was approximately 22 000:1. Even at this relatively high level of competing non-*Salmonella* organisms, *Salmonella* was detected with no apparent difficulty by using one or more of the selective agars. In comparing the overall performance of the 5 agars streaked from SC broth, SS agar gave the lowest number of *Salmonella*-positive samples. An analogous comparison of the agars streaked from TT broth revealed that each agar was able to recover more than 90% of the *Salmonella*-positive samples. One selective enrichment/selective agar combination, TT–XLD, was able to detect *Salmonella* in 100% of the *Salmonella*-positive samples.

Table 7 shows the level of inoculated microorganisms in samples of brown rice. Samples 1–4 were each inoculated with different levels of a lactose-positive *S. arizonae* culture. Samples 1 and 2 were also inoculated with a pool of 4 strains of *E. coli*, whereas samples 3 and 4 were each inoculated with a pool consisting of 2 strains of *Citrobacter*. For reasons discussed previously (see Results and Recommendations, Table 3), this lactose-positive culture was not expected to present an appearance on BG and SS agars that would be considered typical for most *Salmonella* cultures. To be considered typical on HE and XLD, the culture must be able to produce H_2S. Each of various 10-fold dilutions of *S. enteriditis* was inoculated into samples 5–7, all of which also contained added inocula of the *E. coli* pool as described above. This particular strain of *S. enteriditis* produced a distinct off-green colony, rather than the blue or blue-green colony that is typical for *Salmonella* on HE agar.

Sample 8 was inoculated with a pool of 3 *Salmonella* serotypes (*S. muenchen*, *S. cubana*, and *S. saphra*) that was challenged with a pool of 2 strains of *Citrobacter*. Sample 9 contained only *S. hevittingfoss*, whereas sample 10 was the uninoculated negative control.

The results obtained by the collaborators in recovering *Salmonella* from the samples of brown rice are shown in Table 8. A majority of the collaborators were able to recover *S. arizonae* in all or at least a portion of the first 4 samples and BS agar was the favored recovery medium. The isolation of *S. arizonae* on HE and XLD agars was most probably due to the production of H_2S on these 2 agar media. Of the 3 samples (5, 6, 7) inoculated with *S. enteriditis*, one or more of the agars was generally able to isolate *Salmonella* in samples 5 and 6. Only 3 of the collaborators reported the isolation of *Salmonella* in sample 7. This was not too unexpected, however, since it was shown in Table 7 that this sample at the time analysis began had a *Salmonella* MPN of 0.39/g and an *E. coli* MPN of 430/g, representing an *E. coli*/*Salmonella* ratio of approximately 1100:1. Sample 8, containing pools of both *Salmonella* and *Citrobacter*, and sample 9, containing a single unchallenged serotype of *Salmonella*, were positive for *Salmonella* with 1 or more of the agars in all laboratories. Overall, both the BG and the SS agars performed less favorably than the other 3 agars when streaked from either selective enrichment broth. When streaked from the SC broth, the BG and SS agars recovered only 17 and 23%, respectively, of the *Salmonella*-positive samples compared to 56% for the BS agar. Similarly, when streaked from the TT broth, BG and SS agars gave relatively low recoveries of 24 and 25%, respectively, compared to 66 and 65% for the BS and XLD agars, respectively.

The enumeration of inoculated microorganisms in the samples of soy flour is shown in Table 9. Samples 1, 2, and 3 contained each of 10-fold successive dilutions of *S. cubana*, each of which was challenged with the same dilution of a single culture of *Enterobacter*. Samples 4 and 5 contained successive 10-fold dilutions of *S. gaminara*, each sample also containing competing populations of *Enterobacter*. Various 10-fold dilutions of heat-stressed *Salmonella* cells were inoculated into samples 6, 7, and 8 (*S. minnesota*) and sample 9 (*S. sundsvall*).

In milling soy beans into soy flour, any *Salmonella* microorganisms that may be present would be subjected to some degree of heating during processing. Such heating could have a debilitating effect on these bacteria and could ultimately affect the recovery of these organisms on the various selective agars. The effect of nonselective plating media on the recovery of

Table 6. Recovery of *Salmonella* from sunflower seed samples using 5 selective agars [a]

		Selenite cystine					Tetrathionate				
Coll.	Sample No.	BG	BS	SS	HE	XLD	BG	BS	SS	HE	XLD
1[b]	1	+[c]	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
2	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	-	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
3	1	-	+	+	-	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+
	4	-	+	-	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	-	-	-	-	-	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
4	1	E[d]	E	E	E	E	E	E	E	E	E
	2	E	E	E	E	E	E	E	E	E	E
	3	E	E	E	E	E	E	E	E	E	E
	4	E	E	E	E	E	E	E	E	E	E
	5	E	E	E	E	E	E	E	E	E	E
	6	E	E	E	E	E	E	E	E	E	E
	7	E	E	E	E	E	E	E	E	E	E
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
5	1	+	+	-	-	-	+	+	+	+	+
	2	-	+	-	-	-	+	+	+	+	+
	3	+	+	+	+	-	+	+	+	+	+
	4	+	+	-	-	-	+	+	+	+	+
	5	-	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
6	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
7[e]	1	-	-	-	-	-	-	-	-	-	-
	2	+	+	+	+	+	+	+	+	+	+
	3	-	-	-	-	-	-	-	-	-	-
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	-	+	+	+	+	+	+	+
	7	+	+	-	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-

Table 6. (cont'd)

Coll.	Sample No.	Selenite cystine BG	BS	SS	HE	XLD	Tetrathionate BG	BS	SS	HE	XLD
8	1	E	E	E	E	E	E	E	E	E	E
	2	+	+	+	-	-	+	+	-	+	+
	3	+	+	+	+	-	-	-	+	-	+
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	-	+	+	+	+	-	+	+	+	+
	7	+	+	+	+	+	+	+	-	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
9	1	-	+	-	+	+	+	+	-	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	-	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	-	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
10	1	+	+	+	+	+	+	+	-	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	-	+	+
	4	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
11	1	+	+	-	+	-	+	+	+	+	+
	2	+	+	-	+	-	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	-	+	-	+	-	+
	5	+	+	+	+	-	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	-	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
	Totals	60 (90) [f]	66 (99)	54 (81)	61 (91)	56 (84)	64 (96)	65 (97)	62 (93)	65 (97)	67 (100)

[a] For identification of agars, see Table 1, footnotes b-f.

[b] Due to malfunctioning blender, samples 4, 5, 6, 9, and 10 were not blended.

[c] +, Salmonella recovered; -, Salmonella not recovered.

[d] E, data excluded.

[e] Samples 2, 6, 7, 8, and 10 and samples 1, 3, 4, 5, and 9 were initiated 14 and 29 days late, respectively, compared to most other collaborators.

[f] Percentage of total number of 67 positive samples.

Salmonella cells heat-shocked at 55°C for 10 min has been previously reported (12). Accordingly, it was decided to heat-stress the *Salmonella* inocula for 4 of the 10 soy flour samples sent to each collaborator. None of the collaborators experienced any difficulty in recovery of *Salmonella* from samples 6–9 with 1 or more of the selective agars. Analyses of additional samples would be needed, however, before making any definitive conclusions about the relative efficiency of the 5 selective agars for recovery-stressed vs non-stressed *Salmonella* cells from foods.

The recovery of *Salmonella* from the soy flour samples by the collaborators is shown in Table 10. For samples 1–5, which contained both *Salmonella* and *Enterobacter*, the agars streaked from

Table 7. Enumeration of microorganisms in artificially contaminated brown rice samples [a]

Sample No.	Serotype(s)	Dilution[b]	Salmonella MPN/g									
			Selenite cystine broth					Tetrathionate broth				
			BG	BS	SS	HE	XLD	BG	BS	SS	HE	XLD
1	S. arizonae	10^{-3}	< 0.03[e] (< 0.03)	≥3,900 (230)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	≥3,900 (230)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)
2	S. arizonae	10^{-4}	< 0.03 (< 0.03)	430 (930)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	430 (930)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)
3	S. arizonae	10^{-3}	< 0.03 (< 0.03)	430 (2,300)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	430 (2,300)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (0.04)
4	S. arizonae	10^{-4}	< 0.03 (< 0.03)	0.06 (93)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	93 (93)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)
5	S. enteriditis	10^{-2}	ND[f] (2.3)	9,300 (< 0.03)	< 0.03 (< 0.03)	NR[g] NR	9,300 (2.3)	ND (4.3)	≥15,000 (< 0.03)	< 0.03 (< 0.03)	NR NR	≥15,000 (23)
6	S. enteriditis	10^{-3}	430 (< 0.03)	930 (< 0.03)	< 0.03 (< 0.03)	NR NR	430 (< 0.03)	930 (2.3)	930 (< 0.03)	< 0.03 (< 0.03)	NR NR	210 (3.9)
7	S. enteriditis	10^{-4}	23 (< 0.03)	93 (< 0.03)	< 0.03 (< 0.03)	NR NR	150 (< 0.03)	0.2 (0.39)	0.06 (< 0.03)	0.03 (< 0.03)	NR NR	ND (0.39)
8	S. muenchen, S. cubana S. saphra	10^{-4}	< 0.03 (<0.03)	430 (0.06)	< 0.03 (< 0.03)	ND (<0.03)	ND (0.06)	230 (0.75)	930 (43)	930 (4.3)	430 (3.9)	430 (15)
9	S. hevitting-foss	10^{-4}	< 0.03 (< 0.03)	2.1 (0.43)	15 (0.03)	2.1 (0.21)	21 (0.07)	< 0.03 (< 0.03)	93 (23)	93 (23)	93 (23)	93 (23)
10	None	-	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)

Table 7. (cont'd)

Sample No.	Serotype(s)	Citrobacter[c] Dilution[b]	Citrobacter[c] MPN/g Lactose broth BG	BS	SS	HE	XLD	Escherichia coli[d] Dilution[b]	Escherichia coli[d] MPN/g
1	S. arizonae	-						10^{-2}	≥ 230,000 (9,300)
2	S. arizonae	-						10^{-2}	≥930,000 (9,300)
3	S. arizonae	10^{-2}	≥23,000 (≥23,000)	≥ 23,000 (9,300)	≥ 23,000 (≥23,000)	9,300 (≥23,000)	≥ 23,000 (≥23,000)	-	< 0.3 (< 0.3)
4	S. arizonae	10^{-2}	ND (2,300)	≥ 15,000 (4,300)	≥ 43,000 (4,300)	≥ 43,000 (4,300)	ND (1,500)	-	< 0.3 (< 0.3)
5	S. enteriditis	-						10^{-2}	4,300 (≥23,000)
6	S. enteriditis	-						10^{-2}	≥230,000 (≥230,000)
7	S. enteriditis	-						10^{-2}	2,300 (430)
8	S. muenchen, S. cubana S. saphra	10^{-2}	≥230,000 (4,300)	≥230,000 (4,300)	≥230,000 (4,300)	≥230,000 (4,300)	≥230,000 (4,300)	-	< 0.3 (<0.3)
9	S. hevitting-foss	-						-	< 0.3 (< 0.3)
10	None	-						-	< 0.3 (< 0.3)

[a] For identification of agars, see Table 1, footnotes b-f.

[b] One mL of indicated dilution was inoculated into 30 g samples.

[c] Pool consisting of 2 strains of Citrobacter.

[d] Pool consisting of 4 strains of E. coli.

[e] Numbers not in parentheses are MPN values of microorganisms in spiked samples 1 day after inoculation. Numbers in parentheses are MPN values 14 days after inoculation, when actual collaborative study sample analyses were begun.

[f] Positive tube combination too improbable for MPN determination.

[g] Not reported.

Table 8. Recovery of *Salmonella* from brown rice samples using 5 selective agars [a]

	Sample	Selenite cystine					Tetrathionate				
Coll.	No.	BG	BS	SS	HE	XLD	BG	BS	SS	HE	XLD
1	1	[b]	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	+
	7	-	+	+	-	+	+	-	+	-	+
	8	+	+	-	-	-	+	-	-	+	+
	9	-	-	+	-	-	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
2	1	-	-	-	-	+	-	+	-	-	+
	2	-	+	-	+	+	-	+	-	+	+
	3	-	-	-	-	-	-	+	-	-	+
	4	-	-	-	-	-	-	+	-	-	-
	5	+	-	-	+	+	+	-	-	+	+
	6	-	-	-	-	-	-	+	-	+	+
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	+	+	-	+	+
	9	E[c]	E	E	E	E	E	E	E	E	E
	10	-	-	-	-	-	-	-	-	-	-
3	1	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-
	5	-	-	+	-	+	-	-	-	-	+
	6	-	-	-	+	+	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	+	-	-	+	+	+	-	-	+
	9	-	-	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
4	1	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-
	3	E[c]	E	E	E	E	E	E	E	E	E
	4	-	-	-	-	-	-	-	-	-	-
	5	-	+	+	-	+	-	+	+	-	+
	6	-	+	+	-	+	-	+	+	-	+
	7	-	-	-	-	-	-	-	-	-	-
	8	+	+	+	+	+	+	+	+	+	+
	9	-	+	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
5	1	-	+	-	+	+	-	+	-	+	-
	2	-	+	-	-	-	-	+	-	+	-
	3	-	+	-	-	-	-	+	-	-	+
	4	-	+	-	-	-	-	+	-	-	-
	5	-	+	-	-	+	-	+	-	-	-
	6	-	-	-	-	-	-	+	-	-	+
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	+	-	-	+
	9	-	+	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
6	1	-	+	-	-	-	-	+	-	-	-
	2	-	-	-	-	-	-	+	-	-	-
	3	-	+	-	-	-	-	-	-	-	-
	4	-	+	-	-	-	-	+	-	-	-
	5	+	-	-	-	+	-	-	-	-	+
	6	+	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	+	-	-	-	-	-
	8	-	-	-	-	-	+	+	-	+	+
	9	-	+	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
7	1	-	-	-	+	-	-	+	-	+	+
	2	-	+	-	+	+	-	+	-	+	+
	3	-	+	-	+	-	-	+	-	+	+
	4	-	+	-	-	-	-	-	-	-	-
	5	+	-	-	-	+	+	+	-	+	+
	6	-	-	-	-	+	+	+	-	+	+
	7	-	-	-	-	-	-	-	-	-	-
	8	-	+	-	-	+	-	-	+	-	-
	9	-	+	+	+	+	-	-	+	+	+
	10	-	-	-	-	-	-	-	-	-	-

Table 8. (cont'd)

Coll.	Sample No.	Selenite cystine BG	BS	SS	HE	XLD	Tetrathionate BG	BS	SS	HE	XLD
8[d]	1	-	+	-	+	-	-	+	-	+	-
	2	-	-	-	-	-	-	+	-	+	+
	3	-	+	-	+	-	-	+	-	+	+
	4	-	+	-	+	-	-	+	-	-	-
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	+	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	+	-	-	-	+	-	+	-	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
9	1	-	+	+	+	-	-	+	-	+	+
	2	+	+	-	+	+	+	+	-	-	-
	3	-	+	-	+	+	-	+	-	+	+
	4	-	+	-	+	-	+	+	-	+	-
	5	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	+	-	+	-	-	+	-	-	-
	9	-	+	-	+	-	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
10	1	-	+	+	+	+	+	+	+	+	+
	2	+	-	-	+	+	+	+	+	+	+
	3	-	-	-	-	-	-	+	-	-	-
	4	-	-	-	-	-	-	-	-	-	-
	5	+	-	-	-	+	+	-	-	-	+
	6	+	+	-	-	+	-	-	-	-	+
	7	+	-	-	-	+	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	+
	9	-	+	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
11	1	-	-	-	-	-	-	-	-	-	-
	2	-	+	-	+	-	-	-	-	-	-
	3	-	+	-	-	-	-	-	-	-	-
	4	-	+	-	-	-	-	-	-	-	-
	5	-	-	-	+	+	-	-	-	-	+
	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	+
	9	-	-	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
	Totals	12 (17)[e]	40 (56)	16 (23)	29 (41)	35 (49)	17 (24)	47 (66)	18 (25)	32 (45)	46 (65)

[a] For identification of agars, see Table 1, footnotes b-f.

[b] +, Salmonella recovered; -, Salmonella not recovered.

[c] E, data excluded.

[d] Collaborator initiated analyses 1 day late.

[e] Percentage of total number of 71 positive samples.

the TT broth were more productive for *Salmonella* isolations than the analogously paired agars streaked from the SC broth. This was especially evident with the BG agar, where the TT-BG combination recovered *Salmonella* bacteria that, in many instances, were not isolated by the SC-BG combination. The superiority of *Salmonella* recovery on the selective agars streaked from TT broth over those agars streaked from the SC broth was not as evident in samples 6–9, all of which contained heat-stressed *Salmonella* cells. Overall, SS agar streaked from either selective enrichment broth was inferior to the other 4 agars. The SC–SS combination was able to recover *Salmonella* in only 37% of the positive samples compared to 77 and 74% recoverable with the SC–BS and SC–XLD combinations, respectively. Similarly, with the agars streaked from the TT broth, the TT–SS combination recovered the lowest percentage (41%) of *Salmonella*

Table 9. Enumeration of microorganisms in artificially contaminated soy flour samples [a]

Sample No.	Salmonella Serotype(s)	Dilution[b]	MPN/g Selenite cystine broth BG	BS	SS	HE	XLD	MPN/g Tetrathionate broth BG	BS	SS	HE	XLD	Enterobacter aerogenes[c] Dilution[b]	MPN/g
1	S. cubana	10^{-2}	ND[d,e] (1,500)	1,500 (1,500)	120 (ND)	ND (ND)	ND (290)	4,300 (1,500)	4,300 (1,500)	4,300 (< 0.03)	ND (< 0.03)	4,300 (1,500)	10^{-2}	4,300 (930)
2	S. cubana	10^{-3}	ND (230)	430 (230)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	29 (ND)	930 (230)	930 (230)	< 0.03 (0.03)	< 0.03 (< 0.03)	930 (230)	10^{-2}	9,300 (4,300)
3	S. cubana	10^{-4}	< 0.03 (2.1)	4.3 (23)	0.04 (0.04)	< 0.03 (< 0.03)	0.11 (0.75)	93 (23)	93 (23)	< 0.03 (0.03)	0.03 (< 0.03)	43 (23)	10^{-2}	2,300 (1,500)
4	S. gaminara	10^{-3}	0.2 (≥230)	93 (≥230)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	0.34 (93)	230 (≥230)	230 (≥230)	< 0.03 (< 0.03)	0.03 (0.06)	230 (≥230)	10^{-2}	7,500 (930)
5	S. gaminara	10^{-4}	23 (0.23)	43 (0.23)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	0.75 (1.20)	43 (9.3)	43 (9.3)	< 0.03 (< 0.03)	< 0.03 (4.3)	43 (9.3)	10^{-2}	4,300 (930)
6	S. minnesota[f]	10^{-1}	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	230 (23)	-	0.73 (4.3)
7	S. minnesota[f]	10^{-2}	23 (23)	23 (23)	9.3 (23)	9.3 (23)	9.3 (23)	23 (23)	23 (23)	23 (23)	23 (23)	23 (23)	-	0.3 (0.36)
8	S. minnesota[f]	10^{-3}	2.1 (0.93)	9.3 (0.93)	9.3 (0.93)	4.3 (0.93)	9.3 (0.93)	9.3 (0.93)	9.3 (0.93)	9.3 (0.93)	9.3 (0.93)	9.3 (0.93)	-	< 0.3 (< 0.3)
9	S. sundsvall[f]	10^{-2}	≥230 (≥430)	>230 (≥430)	>230 (≥430)	>230 (≥430)	>230 (≥430)	>230 (≥430)	>230 (≥430)	>230 (≥430)	>230 (≥430)	>230 (≥430)	-	< 0.3 (< 0.36)
10	None	-	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	< 0.03 (< 0.03)	-	< 0.3 (< 0.3)

[a] For identification of agars, see Table 1, footnotes b-f.

[b] One-tenth mL of indicated dilution was inoculated into 30 g samples.

[c] Single strain.

[d] Numbers not in parentheses are MPN values of microorganisms in spiked samples 1 day after inoculation. Numbers in parentheses are MPN values 14 days after inoculation, when actual collaborative study sample analyses were begun.

[e] Positive tube combination too improbable for MPN determination.

[f] Heat-shocked.

Table 10. Recovery of *Salmonella* from soy flour samples using 5 selective agars [a]

		Selenite cystine					Tetrathionate				
Coll.	Sample No.	BG	BS	SS	HE	XLD	BG	BS	SS	HE	XLD
1	1	-[b]	+	-	-	-	-	+	+	-	-
	2	-	+	-	-	-	-	+	-	-	-
	3	-	+	-	-	-	+	+	-	-	-
	4	-	-	-	-	-	+	+	-	+	+
	5	-	+	-	+	-	-	+	-	-	-
	6	E[c]	E	E	E	E	E	E	E	E	E
	7	E	E	E	E	E	E	E	E	E	E
	8	E	E	E	E	E	E	E	E	E	E
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
2	1	-	+	-	-	-	+	+	-	+	+
	2	-	+	-	-	+	+	+	-	-	+
	3	+	+	-	-	+	+	+	-	-	+
	4	-	+	-	-	+	+	+	-	+	+
	5	+	+	-	-	+	+	+	-	-	+
	6	+	-	+	-	+	+	+	+	+	+
	7	-	+	-	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
3	1	-	+	-	-	+	+	+	-	-	+
	2	-	+	-	-	+	+	+	-	-	+
	3	-	-	-	-	-	-	+	-	-	+
	4	-	+	-	-	+	+	+	-	+	+
	5	-	+	-	-	+	-	+	-	-	-
	6	+	+	+	+	+	-	+	+	-	+
	7	+	+	+	+	-	+	+	+	+	+
	8	-	-	-	+	-	+	-	-	-	-
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
4	1	-	+	-	+	+	-	+	-	-	+
	2	-	+	-	-	+	-	+	-	-	+
	3	-	+	-	+	+	-	+	-	-	+
	4	-	+	-	+	+	-	+	-	-	+
	5	-	+	-	-	+	-	+	-	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	-	+	-	+	+	-	+	+	-	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
5	1	-	-	-	-	+	-	+	-	-	+
	2	-	+	-	-	+	-	+	-	-	+
	3	-	+	-	-	+	-	+	-	-	+
	4	-	+	-	-	+	+	+	-	+	+
	5	+	+	-	-	+	+	+	-	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	-	+	+	-	+	+	+	+	+	+
	8	-	+	-	-	+	-	-	-	-	-
	9	-	+	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
6	1	-	+	-	-	+	+	+	-	-	+
	2	-	+	-	-	+	+	+	-	-	+
	3	+	+	-	-	+	+	+	-	-	+
	4	-	-	-	-	-	+	+	-	-	+
	5	-	+	-	-	+	-	+	-	-	+
	6	+	+	+	+	+	+	+	+	+	+
	7	-	-	-	-	-	-	+	+	-	-
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
7[d]	1	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	+
	3	-	-	-	-	+	-	+	-	-	+
	4	+	+	-	-	+	-	-	-	-	-
	5	-	+	-	-	+	-	+	-	-	+
	6	+	+	+	+	+	+	+	+	+	+
	7	-	-	+	-	-	-	+	+	+	-
	8	-	-	+	-	-	-	+	+	+	-
	9	+	+	+	+	+	-	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-

Table 10. (cont'd)

Coll.	Sample No.	Selenite cystine BG	BS	SS	HE	XLD	Tetrathionate BG	BS	SS	HE	XLD
8[e]	1	-	+	-	-	+	-	-	-	-	-
	2	-	-	-	-	-	-	+	-	-	+
	3	+	-	-	-	+	+	+	-	-	+
	4	+	+	-	-	+	+	+	-	+	+
	5	+	-	-	-	-	+	+	-	-	+
	6	-	+	+	+	-	-	+	+	+	-
	7	-	-	+	-	-	-	+	+	+	-
	8	+	+	+	+	-	+	+	+	+	-
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
9	1	+	+	-	-	+	+	+	-	-	+
	2	-	+	-	-	+	+	+	-	-	+
	3	+	+	-	-	+	+	+	-	-	+
	4	+	+	-	-	+	+	+	-	-	+
	5	+	+	-	-	+	+	+	-	-	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	-	-	+	-	-	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
10	1	-	-	-	-	+	-	-	-	-	+
	2	+	-	-	-	+	+	-	-	-	+
	3	-	-	-	+	+	-	-	-	+	+
	4	-	-	-	-	-	+	+	-	-	+
	5	-	-	-	-	-	+	+	-	-	+
	6	-	-	-	-	-	-	+	+	-	-
	7	+	+	+	+	-	+	-	-	-	-
	8	+	+	+	-	+	-	+	+	-	+
	9	+	+	+	+	+	+	+	+	+	-
	10	-	-	-	-	-	-	-	-	-	-
11	1	-	+	-	-	+	+	+	-	+	+
	2	-	+	-	-	+	+	+	-	+	+
	3	+	+	-	-	+	+	+	-	+	+
	4	+	+	-	-	-	+	+	-	+	+
	5	-	+	-	-	+	+	+	-	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-	-	-
	Totals	43 (45)[f]	73 (77)	35 (37)	36 (38)	70 (74)	60 (63)	86 (91)	39 (41)	47 (50)	77 (81)

[a] For identification of agars, see Table 1, footnotes b–f.

[b] +, Salmonella recovered; -, Salmonella not recovered.

[c] E, data excluded.

[d] Due to laboratory accident with samples 2, 9, and 10, collaborator re-picked selective agar plates that had been left at room temperature over the weekend.

[e] Collaborator initiated analyses 6 days late.

[f] Percentage of total number of 95 positive samples.

in the positive samples, compared to 91 and 81% by the TT-BS and TT-XLD combinations, respectively.

A statistical pair-wise comparison of the 5 selective agars in their recovery of *Salmonella* from 5 foods was performed (see Collaborative Study). Results are shown in Table 11. With samples of milk chocolate, there was no significant difference between any of the paired combinations of agars streaked from either SC or TT broth. With samples of soy protein powder selectively enriched in SC broth, 4 of the 10 possible comparisons exhibited a significant difference; all 4 of these comparisons demonstrated a significantly

Table 11. Statistical pair-wise comparison of the recovery of *Salmonella* by 5 selective agars

Agar comparison	Milk chocolate		Soy protein powder		Sunflower seeds		Brown rice		Soy flour	
	SC[a]	TT[b]	SC	TT	SC	TT	SC	TT	SC	TT
BG vs BS[c]	—[d]	—	—	—	BS	—	BS	BS	BS	BS
BG vs SS[e]	—	—	BG	BG	BG	—	—	—	—	BG
BG vs HE[f]	—	—	—	HE	—	—	HE	HE	—	BG
BG vs XLD[g]	—	—	—	XLD	—	—	XLD	XLD	XLD	XLD
BS vs SS	—	—	BS	BS	BS	—	BS	BS	BS	BS
BS vs HE	—	—	—	—	—	—	BS	BS	BS	BS
BS vs XLD	—	—	—	—	BS	—	—	—	—	BS
SS vs HE	—	—	HE	HE	HE	—	HE	HE	—	—
SS vs XLD	—	—	XLD	XLD	XLD	XLD	XLD	XLD	XLD	XLD
HE vs XLD	—	—	—	—	—	—	—	XLD	XLD	XLD

[a] Selenite cystine broth.
[b] Tetrathionate broth.
[c] Brilliant green agar vs bismuth sulfite agar.
[d] For a given agar comparison, the agar with a significantly higher ($P < 0.05$) recovery is listed; (—) indicates no significant difference ($P > 0.05$) in their recovery.
[e] *Salmonella-Shigella* agar.
[f] Hektoen enteric agar.
[g] Xylose lysine desoxycholate agar.

lower *Salmonella* recovery with SS agar than with the other 4 agars. When these samples were enriched in TT broth, SS agar again was inferior to the other 4 agars for these same agar comparisons. Additionally, the *Salmonella* recovery obtained by using HE and XLD agars was significantly higher than that obtained with the BG agar. With samples of sunflower seeds selectively enriched in SC broth, 6 of the 10 paired agar combinations exhibited differences in recovering *Salmonella;* 3 of these combinations demonstrated the significantly higher recovery obtained with BS agar than with BG, SS, or XLD agars. In comparing BS and HE agars, no such difference was observed. Of the remaining 3 comparisons showing differences in *Salmonella* recovery, all 3 showed the SS agar to be inferior to the agar to which it was being compared. When the 5 agars were compared for *Salmonella* recovery in samples of sunflower seeds selectively enriched in TT broth, the only significant difference observed was that of XLD agar over SS agar. With the fourth food, brown rice, a relatively large number of significant differences for *Salmonella* recovery in paired agar combinations was observed. For brown rice samples enriched in SC broth, 7 of the 10 agar comparisons showed differences in *Salmonella* recovery. Three of these 7 comparisons showed the BS agar to give a significantly higher *Salmonella* recovery than BG, SS, and HE agars. When the *Salmonella* recovery of the BS agar was compared to that of the XLD agar, no significant difference was observed. Both HE and XLD agars gave a significantly higher recovery of *Salmonella* than BG and SS agars. For samples of brown rice enriched in TT broth, BS, XLD, and HE agars gave significantly higher *Salmonella* recoveries in 3, 3, and 2 of the paired combinations, respectively. In soy flour, the superiority of the BS and XLD agars was evident. When streaked from SC broth, BS and XLD agars each gave significantly higher *Salmonella* recoveries than BG, SS, and HE agars. No significant difference in *Salmonella* recovery was observed between BS and XLD agars. When streaked from TT broth, BS agar gave significantly higher *Salmonella* recoveries than the other 4 selective agars, including the XLD agar. The XLD agar gave a significantly higher *Salmonella* recovery than BG, SS, and HE agars. The BG agar gave a significantly higher *Salmonella* recovery than the SS and HE agars.

Overall, a total of 100 paired combinations of 5 selective agars for recovering *Salmonella* from 5 foods was statistically analyzed. Of this total, BS, XLD, HE, and BG agars gave a significantly higher *Salmonella* recovery rate than the other agar of the pair in 18, 16, 8, and 5 of these comparisons, respectively. In no pair-wise comparison did the SS agar give a significantly higher *Salmonella* recovery than the agar to which it was being compared.

The enumeration of picked cultures that were false positive for *Salmonella* is shown in Table 12. By definition, a false-positive reaction was one in which a culture picked to be *Salmonella* was

Table 12. Enumeration of false-positive cultures using 5 selective agars[a]

		Selenite cystine					Tetrathionate				
Food	Coll.	BG	BS	SS	HE	XLD	BG	BS	SS	HE	XLD
Milk chocolate	1	0/6[b]	0/8	0/6	4/12	0/8	0/6	0/8	0/6	4/12	0/8
	2	4/7	4/6	2/3	4/7	4/7	8/11	6/9	6/9	8/11	8/11
	3	3/6	0/2	0/3	0/3	2/4	2/5	0/3	0/3	0/3	0/3
	4	1/6	2/8	2/8	1/6	0/6	2/7	1/6	0/5	0/5	4/10
	5	0/12	7/16	0/10	0/10	8/16	0/12	4/14	0/10	0/10	6/16
	6	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/0
	7	4/7	3/6	0/3	1/4	7/10	2/5	3/6	0/3	5/8	6/9
	8	4/7	4/7	0/3	3/6	8/10	3/6	5/7	0/3	2/5	6/9
	9	0/4	0/4	0/2	0/2	6/10	0/3	0/3	0/3	0/3	6/9
	10	0/3	0/3	0/1	0/2	2/5	4/7	2/5	2/4	4/7	6/9
	11	0/1	7/8	0/2	0/1	0/1	0/1	4/5	0/2	0/1	0/1
		16/60	27/69	4/42	13/54	37/78	21/64	25/67	8/49	23/66	42/85
	Total[c]	(27)	(39)	(10)	(24)	(47)	(33)	(37)	(16)	(35)	(49)
Soy protein powder	1	0/14	0/14	0/14	0/16	0/14	0/14	0/18	0/14	0/16	0/16
	2	2/9	0/11	1/6	2/9	2/9	2/9	0/11	0/3	2/9	2/9
	3	0/8	0/9	9/12	2/11	3/11	1/9	0/9	9/15	4/12	3/12
	4	0/14	0/4	0/4	0/12	0/12	0/12	0/2	0/4	0/14	0/14
	5	0/14	2/20	2/6	0/9	0/14	0/14	2/20	0/4	0/14	0/16
	6	0/7	0/9	0/1	0/7	0/9	0/7	0/9	0/1	0/8	0/9
	7	1/8	1/10	0/7	2/10	1/10	0/7	0/9	0/5	2/11	2/11
	8	4/11	4/11	2/4	2/9	7/13	4/11	4/11	4/6	2/10	6/13
	9	0/7	0/7	2/9	0/8	2/9	0/7	0/7	2/5	0/8	2/10
	10	0/9	1/10	1/10	1/10	0/9	0/9	0/9	5/9	0/9	0/9
	11	0/7	1/9	2/5	2/9	3/8	0/7	0/9	2/9	3/9	2/9
		7/108	9/114	19/78	11/110	18/118	7/106	6/114	22/75	13/120	17/128
	Total	(7)	(8)	(24)	(10)	(15)	(7)	(5)	(29)	(11)	(13)
Sunflower seeds	1	4/16	2/16	4/18	3/17	0/14	4/18	0/14	4/18	4/18	4/18
	2	2/10	4/11	6/15	4/11	2/10	2/9	3/9	4/12	4/12	2/9
	3	9/13	2/8	9/14	7/12	4/10	5/12	2/9	6/13	6/13	6/13
	4	4/4	0/0	2/2	5/5	4/4	6/6	2/2	2/2	4/4	1/1
	5	2/12	8/18	4/12	4/12	6/12	2/16	6/18	4/18	4/18	2/16
	6	6/13	6/13	6/13	6/13	4/11	6/13	6/13	7/14	6/13	4/11
	7	3/8	9/14	12/16	6/12	7/13	4/9	11/16	5/11	6/13	5/12
	8	2/7	8/15	6/14	4/9	12/16	5/9	7/12	11/16	6/11	8/14
	9	6/12	4/11	6/11	6/13	5/12	5/11	4/11	7/13	6/13	5/12
	10	2/9	0/7	6/13	4/11	6/13	2/9	0/7	10/15	4/11	7/14
	11	2/10	1/11	9/15	6/15	2/5	2/10	1/7	7/15	8/15	1/9
		42/114	44/124	70/143	55/130	52/120	43/122	42/118	67/147	58/141	45/129
	Total	(37)	(36)	(49)	(42)	(43)	(35)	(36)	(46)	(41)	(35)
Brown rice	1	2/4	4/8	12/16	6/6	4/6	6/12	10/12	12/16	4/8	4/12
	2	6/7	15/16	6/6	8/11	11/15	7/9	8/18	8/8	7/12	6/13
	3	8/8	2/3	5/7	6/8	6/10	4/5	0/2	7/8	4/5	7/10
	4	5/6	4/8	3/7	3/5	3/7	4/5	3/7	3/7	5/7	3/7
	5	0/0	12/20	2/4	6/10	14/18	0/0	10/20	6/8	10/15	16/20
	6	3/5	12/16	2/3	4/5	0/3	5/6	10/15	4/5	7/9	4/7
	7	1/1	10/15	9/10	6/10	10/15	3/5	13/18	1/3	5/11	4/10
	8	12/13	7/13	10/11	5/11	16/18	9/11	8/17	12/14	8/15	15/19
	9	12/14	7/13	14/15	2/8	14/16	10/12	9/15	15/16	8/14	14/17
	10	6/10	5/8	6/8	3/6	3/9	5/8	3/7	0/3	2/5	3/9
	11	12/12	14/18	15/16	13/16	2/4	11/11	17/18	16/17	16/17	1/6
		67/80	92/138	84/103	62/96	83/121	64/84	91/149	84/105	76/118	77/130
	Total	(84)	(67)	(82)	(65)	(69)	(76)	(61)	(80)	(64)	(59)
Soy flour	1	0/2	0/10	0/2	0/4	0/2	0/6	0/12	0/4	0/4	0/4
	2	3/8	5/13	3/6	4/7	5/13	3/12	4/13	0/4	2/8	3/12
	3	1/4	1/8	0/3	1/5	0/6	0/6	2/10	0/3	2/5	0/7
	4	0/3	0/9	0/3	0/7	0/9	0/2	0/9	0/4	0/4	0/9
	5	0/3	7/16	0/4	0/3	0/15	0/6	7/16	0/4	0/8	0/14
	6	2/6	6/13	5/8	2/5	0/7	2/9	0/9	2/6	2/5	0/8
	7	2/5	10/14	2/6	2/4	5/10	1/2	7/13	0/4	1/5	6/11
	8	1/6	2/7	3/7	2/5	12/16	0/5	1/10	0/4	0/5	9/14
	9	0/7	7/15	3/7	0/3	9/17	0/9	5/14	2/6	0/4	9/18
	10	2/6	0/3	1/4	0/3	4/9	3/8	0/5	2/5	0/2	5/11
	11	0/6	4/13	1/5	0/4	2/10	0/9	3/12	0/4	2/11	3/12
		11/56	42/121	18/55	11/50	37/114	9/74	29/123	6/48	9/61	35/120
	Total	(20)	(35)	(33)	(22)	(33)	(12)	(24)	(13)	(15)	(29)

[a] For identification of agars, see Table 1, footnotes *b–f*.

[b] Each numerical entry represents the number of colonies picked that were not *Salmonella* (numerator) divided by the number of colonies that could be identified as *Salmonella* or non-*Salmonella* (denominator).

Table 13. Statistical pair-wise comparison of false-positive reactions for *Salmonella* enumerated by 5 selective agars

Agar comparison	Milk chocolate		Soy protein powder		Sunflower seeds		Brown rice		Soy flour	
	SC[a]	TT[b]	SC	TT	SC	TT	SC	TT	SC	TT
BG vs BS[c]	—[d]	—	—	—	—	—	BG	—	—	—
BG vs SS[e]	—	—	SS	—	—	—	—	—	—	—
BG vs HE[f]	—	—	—	—	—	—	BG	—	—	—
BG vs XLD[g]	—	—	—	—	—	—	—	—	—	—
BS vs SS	BS	—	SS	SS	—	—	SS	SS	—	—
BS vs HE	—	—	—	—	—	—	—	—	—	—
BS vs XLD	—	—	—	—	—	—	—	—	—	—
SS vs HE	—	—	—	—	—	—	SS	SS	—	—
SS vs XLD	XLD	XLD	—	—	—	—	—	SS	—	—
HE vs XLD	—	—	—	—	—	—	—	—	—	—

[a] Selenite cystine broth.
[b] Tetrathionate broth.
[c] Brilliant green agar vs bismuth sulfite agar.
[d] For a given agar comparison, the agar giving a significantly higher ($P < 0.05$) enumeration of false-positive reactions is listed; (—) indicates no significant difference ($P > 0.05$) in the enumeration of false-positive reactions.
[e] *Salmonella-Shigella* agar.
[f] Hektoen enteric agar.
[g] Xylose lysine desoxycholate agar.

shown to be non-*Salmonella* by subsequent biochemical and serological testing. For entries in this table, the numerator gives the actual number of false-positive cultures picked and the denominator gives the total number of picked cultures which could be reasonably identified either as *Salmonella* or non-*Salmonella*. Picked cultures in which an insufficient number of biochemical and/or serological tests were performed were not included in the calculations. The false-positive rate (FPR), expressed as a percentage, is the ratio of false-positive cultures picked to the total number of picked cultures which could be identified for any particular food.

For the milk chocolate samples, SS agar, streaked from either selective enrichment, gave the lowest FPR value, XLD agar the highest. The high selectivity of SS agar has been reported by other workers. In comparing the efficiency of SS, HE, and XLD agars for recovering *Salmonella* and *Shigella* from clinical specimens, Pollock and Dahlgren (13) reported that SS agar was more inhibitory and recovered significantly fewer positive samples than did HE and XLD agars, and that both HE and XLD agars avoided the combination of bile salts and citrate thought to be responsible for the low plating efficiency of certain strains of *Shigella* and *Salmonella* on SS agar. Despite its high selectivity, SS agar is generally acknowledged to have a poor differential system, so that it is often difficult to distinguish *Salmonella* from suspicious non-*Salmonella* colonies. Pollock and Dahlgren (13) found that *Proteus*, late lactose-fermenting *E. coli*, and *Pseudomonas* accounted for most of the false-positive picks on SS agars.

For samples of soy protein powder, sunflower seeds, and brown rice in Table 12, SS agar gave FPR values higher than those of the other 4 agars when streaked from either SS or TT broth (except for BG agar streaked from SC-enriched samples of brown rice). With samples of soy flour, BS agar and XLD agar gave the highest FPR values for agars streaked from SC and TT broths, respectively. It should be pointed out that with the AOAC method (2), BG and SS agar plates, in addition to plates of BS agar, should be incubated an additional 24 h if no typical or suspicious *Salmonella* colonies are present after the initial 24 h incubation. This practice is especially valuable in the case of SS agar where many late lactose-fermenting cultures, appearing typical after 24 h of incubation, may appear nontypical after an additional 24 h incubation period. Nevertheless, the manufacturer's instructions (14) stated that the SS agar should be incubated at 35–37°C for a full 24 h and the BG agar at 37°C for 18–24 h. For the purpose of this study, collaborators were instructed to incubate all selective agars at 35°C for 24 ± 2 h, with the exception of the BS agar, which was incubated an additional 24 ± 2 h if the BS agar plates did not have typical or suspicious *Salmonella* colonies or did not contain growth after the initial 24 h incubation.

In Table 13 are shown the results of a statistical pair-wise comparison of FPR values (see Collaborative Study) given by the 5 selective agars.

Table 14. Enumeration of false-negative reactions using 5 selective agars[a]

		Selenite cystine					Tetrathionate				
Food	Coll.	BG	BS	SS	HE	XLD	BG	BS	SS	HE	XLD
Milk chocolate	1	1	0	1	0	0	0	0	1	0	0
	2	0	1	2	0	0	0	0	0	0	0
	3	0	1	0	0	1	0	0	0	0	0
	4	0	0	0	0	0	0	0	1	0	0
	5	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	1
	7	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	1	0	0	0	0	0
	9	0	0	1	1	0	0	0	0	0	0
	10	0	0	2	1	0	0	0	1	0	0
	11	1	1	0	1	1	1	1	0	1	1
	Total	2	3	6	3	3	1	1	3	1	2
Soy protein powder	1	2	2	2	1	2	2	0	2	1	1
	2	2	0	4	2	2	2	0	6	2	2
	3	1	0	4	0	1	1	0	3	1	0
	4	0	5	5	1	1	1	6	5	0	0
	5	2	0	7	3	2	2	0	7	2	1
	6	2	0	8	2	0	2	0	8	1	0
	7	2	0	2	1	0	2	0	4	0	0
	8	2	2	7	2	3	2	2	7	1	2
	9	1	1	1	0	1	1	1	5	0	0
	10	0	0	0	0	0	0	0	5	0	0
	11	2	1	6	2	4	2	0	2	3	2
	Total	16	11	46	14	16	17	9	54	11	8
Sunflower seeds	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	1	0	0	0	0	0	0	0
	3	3	1	2	2	1	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
	5	2	0	3	3	4	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0
	7	0	0	2	0	0	0	0	0	0	0
	8	1	0	0	1	2	2	1	2	1	0
	9	1	0	2	0	0	1	0	1	0	0
	10	0	0	0	0	0	0	0	2	0	0
	11	0	0	3	0	4	0	1	0	1	0
	Total	7	1	13	6	11	3	2	5	2	0
Brown rice	1	3	2	2	4	3	1	3	2	2	0
	2	6	6	7	5	4	5	1	7	3	1
	3	4	3	2	2	0	3	2	3	3	1
	4	3	0	0	2	0	3	0	0	2	0
	5	8	2	7	6	5	8	0	7	5	4
	6	7	5	8	8	6	8	4	8	7	6
	7	7	3	7	4	3	6	3	6	2	2
	8	6	2	6	3	5	5	2	5	3	3
	9	5	0	5	0	4	4	0	5	2	3
	10	4	5	6	5	2	5	4	5	5	2
	11	6	3	5	3	4	6	5	5	5	3
	Total	59	31	55	42	36	54	24	53	39	25
Soy flour	1	5	1	5	4	5	3	0	4	4	4
	2	4	1	6	6	1	0	0	5	3	0
	3	6	2	6	5	3	3	1	6	6	2
	4	6	0	6	2	0	7	0	5	5	0
	5	7	1	6	7	0	5	1	6	4	1
	6	5	2	6	6	2	2	0	5	6	1
	7	5	4	4	6	3	7	2	4	4	3
	8	4	4	5	6	5	4	1	5	4	4
	9	2	1	5	6	1	0	0	5	5	0
	10	5	6	6	6	4	4	4	6	7	3
	11	3	0	5	5	1	0	0	5	0	0
	Total	52	22	60	59	25	35	9	56	48	18

[a] For identification of agars, see Table 1, footnotes *b–f*.

Table 15. Statistical pair-wise comparison of false-negative reactions for *Salmonella* given by 5 selective agars

Agar comparison	Milk chocolate		Soy protein powder		Sunflower seeds		Brown rice		Soy flour	
	SC[a]	TT[b]	SC	TT	SC	TT	SC	TT	SC	TT
BG vs BS[c]	—[d]	—	—	—	BG	—	BG	BG	BG	BG
BG vs SS[e]	—	—	SS	SS	—	—	—	—	—	SS
BG vs HE[f]	—	—	—	—	—	—	—	—	—	—
BG vs XLD[g]	—	—	—	—	—	—	BG	BG	BG	BG
BS vs SS	—	—	SS	SS	SS	—	SS	SS	SS	SS
BS vs HE	—	—	—	—	—	—	—	—	HE	HE
BS vs XLD	—	—	—	—	XLD	—	—	—	—	—
SS vs HE	—	—	SS	SS	—	—	—	—	—	—
SS vs XLD	—	—	SS	SS	—	SS	SS	SS	SS	SS
HE vs XLD	—	—	—	—	—	—	—	—	HE	HE

[a] Selenite cystine broth.
[b] Tetrathionate broth.
[c] Brilliant green agar vs bismuth sulfite agar.
[d] For a given agar comparison, the agar giving a significantly higher ($P<0.05$) enumeration of false-negative reactions is listed; (—) indicates no significant difference ($P>0.05$) in the enumeration false-negative reactions.
[e] *Salmonella-Shigella* agar.
[f] Hektoen enteric agar.
[g] Xylose lysine desoxycholate agar.

With 2 of the foods, sunflower seeds and soy flour, there was no significant difference in the FPR values in any of the paired combinations of selective agars. For the remaining 3 foods, in 13 instances 1 of the agars gave a significantly higher rate of false-positive cultures than the agar to which it was being compared. Of the 13 instances, a higher FPR value was given with the SS agar in 8 comparisons; BG agar (2 comparisons); XLD agar (2 comparisons); and the BS agar (1 comparison).

Table 14 shows the number of false-negative reactions by the 5 selective agars. By definition, a false-negative reaction was one in which any particular selective enrichment broth/agar combination did not detect *Salmonella* that was detected by 1 or more of the other 9 enrichment/agar combinations. In the event that all 5 agars streaked from 1 enrichment were all negative and *Salmonella* was detected on 1 or more agars streaked from the other enrichment, then all 5 agars streaked from the first enrichment were considered to be false negatives by definition. There is the possibility, however, that there were no viable *Salmonella* bacteria in that particular selective enrichment from which all the streaked agars were negative, a possibility which cannot be determined. As long as the data are treated consistently, however, the introduction of any bias with respect to the definition of a false-negative reaction should be minimal. SS agar streaked from SC broth and TT broth gave the highest number of false-negative reactions with 2 exceptions (BG agar streaked from SC or TT enrichments of the brown rice samples). In contrast, BS agar, with a few exceptions, gave numbers of false-negative reactions that were lower than those of the other selective agars streaked from a given selective enrichment. One reason for the relatively low enumeration of false-negative reactions by the BS agar was the ability of this agar to detect lactose-positive *Salmonella* organisms (Tables 4 and 8) that in many instances went undetected by the other agars. That some collaborators detected lactose-positive cultures on agars other than BS agars could be due either to analysts picking nontypical *Salmonella* colonies on 1 or more of the non-BS agars, or to differences in human judgment as to what is meant by the designation "typical."

Table 15 reports the results of a statistical pair-wise comparison of false-negative reactions (see Collaborative Study) enumerated by the 5 selective agars. With samples of milk chocolate, there was no significant difference in the enumeration of false-negative reactions in any of the paired agar combinations. For the remaining 4 foods, in 33 instances 1 member of a paired combination of agars gave a significantly higher number of false-negative reactions than the other member. Of these 33, a higher enumeration of false-negative reactions was given with the SS agar (19 comparisons), BG agar (9 comparisons),

Table 16. Summary of statistical pair-wise comparison of 5 selective agars according to 3 parameters

Agar[a]	Parameter[b]		
	Productivity	False-positive rate	False negatives
BG	5	2	9
BS	18	1	0
SS	0	8	19
HE	8	0	4
XLD	16	2	1

[a] For identification of agars, see Table 1, footnotes *b–f*.

[b] Each numerical entry represents the total number of times for all 5 foods combined that any particular agar of a paired agar combination gave a significantly higher number of responses than did the member to which it was compared.

HE agar (4 comparisons), and XLD agar (1 comparison). The results of the statistical analysis of the data in Tables 11, 13, and 15 are summarized in Table 16. The efficiency of the agars was statistically compared according to 3 parameters: (*1*) productivity or recovery of *Salmonella*, (*2*) rate of enumeration of cultures that were false positive for *Salmonella*, and (*3*) the rate of false-negative reactions. Overall, it appears that SS agar, because of its relatively low productivity and its high rate of enumeration of false-positive and false-negative reactions, could be excluded from the series of selective agars without significantly reducing the detection rate of *Salmonella* in the food examined in this study. BS and XLD agars were the most productive for *Salmonella* recovery while having rates of false-positive and false-negative reactions that were low compared to the rates of these reactions enumerated by the other 3 agars. Thus, the decision seems to be whether 1 or both of the remaining 2 agars, HE and BG, should be included in the analytical protocol. A re-examination of Table 11 reveals that when HE and BG agars were compared with respect to productivity, HE agar recovered a significantly higher number of *Salmonella*-positive samples in 3 of 10 comparisons, and BG agar recovered a significantly higher number of *Salmonella*-positive samples in only 1 of these comparisons. A review of Table 13 shows that where BG and HE agars were compared for rates of enumeration of false-positive cultures, all comparisons showed equivalency for the paired agars, except for a significantly higher false-positive rate obtained on BG agar, compared to HE agar, when streaked from SC-enriched samples of brown rice. Finally, in Table 15, it can be seen that where HE and BG agars are compared for the enumeration of false-negative reactions, they were equivalent in all 10 comparisons. Accordingly, using the parameters as defined above, use of HE agar appeared to have some slight advantage over BG agar in this collaborative study.

In a 2½-year comparison of HE, BG, BS, and SS agars for recovering *Salmonella* from naturally contaminated food and feed samples, Bisciello and Schrade (15) reported that the greatest number of *Salmonella*-positive samples were obtained with HE agar and that this agar also gave the lowest number of false-positive cultures. Moreover, of a total of 288 *Salmonella*-positive samples, *Salmonella* would have gone undetected in 20 samples if a combination of only BG, BS, and SS agars had been used.

At the conclusion of the collaborative study, the participants were asked to indicate their relative preferences for the 5 agars. Of the 15 analysts or analyst teams responding, BS agar was the first choice of 6 analysts; HE of 4 analysts; XLD of 3 analysts; and BG of 2 analysts. Conversely, SS agar was the least favored of 12 analysts and BS, XLD, and BG were the least favored by 1 analyst each.

The results of this study have demonstrated that of the 5 selective agars compared with respect to 3 parameters, BS and XLD agars were the most efficient and SS agar was the least efficient. The other 2 agars, HE and BG, were intermediate in their efficiency, with the efficiency of the HE agar somewhat greater than that of the BG agar, but not decisively. Accordingly, it is recommended that the official final action method for the detection of *Salmonella*, **46.054-46.067,** be revised to include addition of XLD and HE agars and deletion of SS and BG agars. The following changes are required:

(a) In **46.054,** delete the entire sections (d) *Brilliant green agar* and (e) *Salmonella-Shigella agar.* Substitute for them the following sections:

(d) *Xylose lysine desoxycholate agar (XLD).*— Suspend ingredients (*1*) or (*2*) (varies with mfgr of formula) in 1 L H_2O and mix thoroly. Heat with frequent agitation just until medium boils. Do not overheat. Cool in H_2O bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.4 ± 0.2. Do not autoclave.

(*1*) 3.5 g xylose, 5.0 g L-lysine, 7.5 g lactose, 7.5 g sucrose, 5.0 g NaCl, 3.0 g yeast ext, 0.08 g phenol red, 2.5 g Na desoxycholate, 6.8 g Na thiosulfate, 0.8 g ferric ammonium citrate, and 13.5 g agar.

(2) 3.75 g xylose, 5.0 g L-lysine, 7.5 g lactose, 7.5 g sucrose, 5.0 g NaCl, 3.0 g yeast ext, 0.08 g phenol red, 2.5 g Na desoxycholate, 6.8 g Na thiosulfate, 0.8 g ferric ammonium citrate, and 15 g agar.

(e) *Hektoen enteric agar (HE).*—Suspend ingredients (1) or (2) (varies with mfgr of formula) in 1 L H_2O and mix thoroly. Heat to boiling with frequent agitation and let boil few moments. Do not overheat. Cool in H_2O bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.6 ± 0.2. Do not autoclave.

(1) 12.0 g thiotone peptone, 3.0 g yeast ext, 9.0 g bile salts, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.064 g bromthymol blue, 0.1 g acid fuchsin, and 13.5 g agar.

(2) 12.0 g proteose peptone, 3.0 g yeast ext, 9.0 g bile salts No. 3, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.065 g thymol blue, 0.1 g acid fuchsin, and 14.0 g agar.

(b) In **46.057,** substitute the following sections:

(a) *Growth in selective broth.*—Gently shake incubated sample mixt., **46.056,** and transfer 1 mL to 10 mL selenite cystine broth, **46.054(b)** (1) or (2), and addnl 1 mL to 10 mL tetrathionate broth, **46.054**(c). Incubate 24 ± 2 h at 35°. (For dried active yeast, substitute lauryl sulfate tryptose broth, **46.013(b)**, for selenite cystine broth, **46.054(b)** (1) or (2).

Streak 3 mm loopful of incubated selenite cystine broth on selective media plates of xylose lysine desoxycholate agar, **46.054(d)**, Hektoen enteric agar, **46.054**(e), and $Bi_2(SO_3)_3$ agar, **46.054(f)**. Repeat with 3 mm loopful of incubated tetrathionate broth. Incubate plates 24 ± 2 h at 35°.

(b) *Appearance of typical Salmonella colonies.*—(1) *On xylose lysine desoxycholate agar.*—Pink colonies with or without black centers. Many *Salmonella* may have large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* cultures produce yellow colonies with or without black centers.

(2) *On Hektoen enteric agar.*—Blue-green to blue colonies with or without black centers. Many *Salmonella* cultures may have large glossy black centers or may appear as almost completely black colonies.

(3) *On bismuth sulfite agar.*—Brown, gray, or black, sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time. Some strains produce green colonies with little or no darkening of surrounding medium.

Examine XLD and HE agar plates for typical or suspicious *Salmonella* colonies after 24 ± 2 h incubation at 35°. BS agar plates should be examined for typical or suspicious *Salmonella* colonies after 24 ± 2 h and 48 ± 2 h incubation at 35°.

(c) In **46.058**(a) in the 1st sentence, substitute "xylose lysine desoxycholate, Hektoen enteric" for "brilliant green, *Salmonella-Shigella* . . ."

(d) In **46.059,** delete the entire section (**b**) and substitute the following:

(**b**) *Mixed cultures.*—Streak any culture that appears to be mixed on MacConkey agar, **46.054(q)**, or xylose lysine desoxycholate agar, **46.054(d)**, or Hektoen enteric agar, **46.054(e)**. Incubate 24 ± 2 h at 35°.

Delete (c)(2) and substitute:

(2) *On xylose lysine desoxycholate agar.*—See **46.057**(b)(1).

As the next sentence after (c)(2) add:

(3) *On Hektoen enteric agar.*—See **46.057(b)**-(2).

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FOOD ADDITIVES

Rapid Method for Estimation of *N*-Nitrosodimethylamine in Malt Beverages

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A new, rapid column elution method is described for the estimation of *N*-nitrosodimethylamine in malt beverages. Recoveries for *N*-nitrosodimethylamine and *N*-nitrosodipropylamine fortified at 5 ppb averaged 90 and 91%, respectively. A study of 20 different malt beverages showed that the resuts obtained with this method compared favorably with those obtained by a direct distillation procedure.

Reports in 1978 that a majority of beers available in West Germany contained traces of *N*-nitrosodimethylamine (NDMA) have stimulated a number of investigations into the presence of this animal carcinogen in malt beverages (1). Spiegelhalder et al. (2) estimated that 64% of a West German male's dietary exposure to NDMA is from consumption of beer. Subsequent reports have indicated that a majority of malt beverages, regardless of origin, contain NDMA. These findings have prompted the U.S. Food and Drug Administration (FDA) to establish a regulatory action level of >5 ppb NDMA in malt beverages.

Nearly all recent analytical methods have used a gas chromatographic-thermal energy analyzer (GC-TEA) for detection and quantitation of NDMA in malt beverages. A variety of procedures have, however, been used to isolate volatile nitrosamines from malt beverages. Spiegelhalder et al. (1) vacuum-distilled beer, partitioned the distillate with dichloromethane (DCM), and concentrated the solvent in a Kuderna-Danish (K-D) evaporative concentrator. Walker et al. (3) directly extracted alcoholic beverages with DCM, then centrifuged the extract and concentrated it in a K-D apparatus. Goff and Fine (4) used liquid-liquid partition of beers in a commercial apparatus specially designed for nitrosamines (Preptube) followed by concentration in a K-D apparatus. Scanlan et al. (5) analyzed several U.S.-produced beers by steam distillation followed by extraction of the distillate with DCM. Sen et al. (6) vacuum-distilled beers in a flash evaporator after adding aqueous base, then extracted the distillate with DCM, washed it, and concentrated it in a K-D apparatus. We surveyed a large number of domestic and imported beers by using an atmospheric distillation in which the malt beverage was first saturated with solid $Ba(OH)_2$ (7), and the aqueous distillate was then extracted with DCM and concentrated in a K-D apparatus. While all these procedures appear workable, none completely suited our need for producing acceptable data with speed, simplicity, and efficiency.

We report here a simple method which produces data comparable to our previous method (7) in less time and with less operator involvement. The method is similar to that used by Fiddler et al. (USDA (1980), private communication) for the determination of nitrosopyrrolidine in fried bacon.

METHOD

Reagents

(a) *Celite® 545 (not acid-washed).*—Fisher Scientific Co., Pittsburgh, PA 15219 (No. C-212). Heat contents of each bottle 16 h at 700°C before use.

(b) *Dichloromethane (DCM).*—Glass-distilled (Burdick and Jackson Laboratories, Muskegon, MI 49442).

(c) *Sodium sulfate.*—Anhydrous granular, reagent grade (Mallinckrodt Inc., Paris, KY 40361).

(d) *Ethanol.*—Anhydrous (National Distillers and Chemical Corp., New York, NY 10016).

(e) N-*Nitrosodimethylamine (NDMA).*—Serially dilute previously prepared stock solution (2 mg/mL) to working standard (0.5 μg/mL) with DCM.

(f) N-*Nitrosodipropylamine (NDPA).*—Serially dilute previously prepared stock solution to 0.25 μg/mL with anhydrous ethanol. (Caution: NDMA and NDPA are potent animal carcinogens and must be handled appropriately.)

(g) *Boiling chips.*—Carborundum, small size (or equivalent).

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Ensure absence of positive responses for each new lot of reagent by running blanks. Use 25 mL 4% ethanol in distilled (not deionized) water in place of malt beverage.

Apparatus

(a) *Chromatographic column.*—Glass, 28 mm id × 400 mm long with stopcock.

(b) *Evaporative concentrator.*—Kuderna-Danish, 250 mL with 4 mL concentrator tube and 3-ball distilling column (Kontes Glass, Vineland, NJ 08360, K-503000-0121, K-570001-0250, K-570050-425).

(c) *Tamping rod.*—19 mm diameter disc.

(d) *Glass wool.*—Pyrex or equivalent.

(e) *Gas chromatograph-thermal energy analyzer.*—Hewlett-Packard Model 5710A GC interfaced to a TEA Model 502L. Instrumental conditions: 2.7 m × 4 mm id glass column packed with 10% Carbowax® 1540 and 5% KOH on 100–120 mesh Chromosorb® WHP; carrier gas, argon, 40 mL/min; temperatures (°C), injector 200, column 150, TEA furnace 450; pressure 1.2 torr; liquid nitrogen cold trap.

Thoroughly clean all glassware, including a chromic acid rinse, before each analysis.

Determination

Weigh 25.0 ± 0.1 g malt beverage into tared 600 mL beaker. Add 125 ng (0.5 mL) NDPA and 25 g Celite. Stir mixture until uniform (ca 30 s). Mixture will not pour but will appear light and fluffy. Place small glass wool plug in bottom of column and cover with 20 g Na_2SO_4. Place tamping rod and powder funnel in column with end of the tamping rod extending through funnel opening. With spatula, load beer-Celite mixture into column and tamp mixture, a little at a time, to depth of 8–10 cm. Add 75 mL DCM to beaker, swirl with spatula, and pour through funnel before removing tamping rod. Adjust stopcock so DCM flows at rate of 1–2 mL/min into K-D flask fitted with 4 mL concentrator tube. Let column run dry. Approximately 35 mL DCM will be recovered.

Add 3 small boiling chips, fit K-D with distilling column, and concentrate DCM to ca 4 mL in 60°C water bath. Let column drain and further concentrate DCM to 1.0 mL under stream of nitrogen at ambient temperature. Make duplicate 8 μL injections into GC-TEA and quantitate any peaks with proper retention times against duplicate external standards. Calculate NDMA concentration and % NDPA recovery on basis of average peak heights. If less than 85% of the NDPA is recovered, repeat analysis.

Table 1. Recovery of NDMA and NDPA from beer spiked at 5 ppb

Run No.	NDMA rec., %	NDPA rec., %
1	91	94
2	95	95
3	93	91
4	91	96
5	85	91
6	89	91
7	86	87
8	90	92
9	90	89
10	90	88
Range	85–95	87–96
$\bar{x}$	90.0	91.4
SD	2.94	2.95

Results and Discussion

To determine the percent recovery of the column elution method, 125 ng each of NDMA and NDPA were added to 25 g beer which had given a negative response for nitrosamines (Table 1), and the spiked samples were analyzed by the procedure. Average NDMA and NDPA recoveries were 90 and 91%, respectively.

The recovery of NDMA reported here is the same as that obtained by an atmospheric distillation technique (7) and 19% higher than that reported for a vacuum distillation method (6). Scanlan et al. (5) reported an NDMA recovery of approximately 75% for a steam distillation method, and Goff and Fine (4) obtained recoveries ranging from 45 to 62%, using the Preptube method.

To investigate the linearity of the assay over the range of NDMA concentrations expected, a blank beer was spiked with NDMA and NDPA at 6 concentrations ranging from 0.1 to 15 ppb. The method proved to be nearly linear (coefficient of determination, $r^2 = 0.998$ and 0.997 for NDMA and NDPA, respectively). The slope of the NDPA line was slightly closer to unity, indicating a somewhat higher recovery of NDPA than NDMA.

The precision of the method was tested by analyzing 10 replicates of a single can of beer which was known to contain just under 5 ppb NDMA (Table 2). All analyses were performed by the same analyst during the same day. The uncorrected recovery mean concentration was 4.28 ppb with a standard deviation (SD) of 0.07. Correction for NDPA recovery increased these values to 4.68 ppb and 0.11, respectively. The method had a detection limit of 0.1 ppb.

Both uncorrected and corrected values have been reported for NDMA in malt beverages, al-

Table 2. Replicate analyses of a single beer sample

Run No.	NDPA rec., %	Uncorrected NDMA, ppb	Corrected NDMA, ppb
1	96	4.39	4.57
2	89	4.23	4.75
3	91	4.31	4.74
4	93	4.23	4.55
5	88	4.28	4.86
6	90	4.21	4.68
7	87	4.21	4.84
8	95	4.34	4.57
9	90	4.20	4.67
10	95	4.38	4.61
Range	87–96	4.20–4.39	4.55–4.86
$\bar{x}$	91.4	4.28	4.68
SD	3.17	0.07	0.11

though a majority of workers add an internal standard. Walker et al. (3) reported corrected values using *N*-nitrosomethylpentylamine as an internal standard; Sen et al. (6) reported uncorrected values and gave an average percent recovery of NDMA. Spiegelhalder et al. (1) added an internal standard (NDPA) but it is unclear whether their data were corrected for recovery. Our data (Table 2) indicate that correction for losses using NDPA as an internal standard may slightly decrease the precision of the analysis. This may be due to the less than perfect correlation coefficient between the recoveries of NDMA and NDPA, namely, 0.56 (Table 1).

The column elution method was compared with the distillation procedure we described earlier (7). Twenty different malt beverages, collected during a recent FDA survey, were analyzed by the 2 methods. Each sample was analyzed by both methods on the same day by the same analyst. The column elution method gave slightly higher uncorrected NDMA concentrations in 13 of 20 analyses, equal concentrations in 5 of 20 analyses, and lower concentrations in 2 of 20 samples (Table 3); the method averaged 0.18 ppb higher for these 20 samples. Overall, the 2 methods compared favorably.

Samples 9 and 15 were not amenable to direct distillation and had to be steam-distilled because of the formation of a stable foam. Sample 9 was a high specific gravity nonalcoholic malt beverage and Sample 15 an artificially colored and flavored malt liquor. Both samples were amenable to the column elution method.

The potential of the column elution method to form nitrosamines as an artifact of the procedure was investigated by adding 10 ppm each of morpholine and nitrite to a beer that had given a negative response for nitrosamines and immediately analyzing the mixture. Neither NDMA nor nitrosomorpholine was found in the sample.

The column elution method has certain advantages over other published methods. Be-

Table 3. Comparative analyses of several different malt beverages

		Column			Distillation		
Sample	Type	NDPA rec., %	Uncorrected NDMA, ppb	Corrected NDMA, ppb	NDPA rec., %	Uncorrected NDMA, ppb	Corrected NDMA, ppb
1	Ale	93	13.3	14.3	94	12.6	13.4
2	Lager	94	2.0	2.1	95	1.7	1.8
3	Dark	95	6.9	7.3	96	6.6	6.9
4	Lager	96	0.3	0.3	96	0.3	0.3
5	Lager	100	2.9	2.9	100	2.6	2.6
6	Lager	98	7.8	8.0	99	7.3	7.4
7	Lager	96	5.5	5.7	100	5.4	5.4
8	Lager	95	2.3	2.4	95	2.0	2.1
9	Nonalcoholic	92	4.1	4.5	94	3.5[a]	3.7
10	Dark	98	8.3	8.5	106	8.4	7.9
11	Lager	98	2.1	2.1	96	2.0	2.1
12	Light	98	0.8	0.8	99	0.8	0.8
13	Lager	98	9.3	9.5	100	9.5	9.5
14	Lager	90	4.1	4.6	95	4.1	4.3
15	Flavored	94	0.2	0.2	94	0.2[a]	0.2
16	Dark	98	1.0	1.0	93	0.9	1.0
17	Dark	90	1.0	1.1	90	0.9	1.0
18	Lager	89	ND[b]	ND	89	ND	ND
19	Lager	93	2.2	2.4	94	2.1	2.2
20	Lager	96	8.2	8.5	92	7.9	8.6

[a] By steam distillation.
[b] ND = not detected.

cause the method does not involve any type of distillation or centrifugation, the amount and complexity of the necessary equipment is greatly reduced as well as the need to purchase specially designed products. The amount of analyst's time and attention required is not great; thus one analyst can complete 8–12 analyses/day including cleaning of glassware. Also, the column elution technique will work for all types of malt beverages encountered, whereas direct distillation does not.

Analyses by GC-MS of a further concentrated extract (100 μL) of the column eluate showed a number of interfering compounds near the retention time of NDMA. Mass spectral confirmation may be more easily achieved by previously published methods (7). Other work in our laboratory supports the use of a modification of this technique for the analysis of volatile *N*-nitrosamines in certain dry products as well as liquids. These areas will be the subject of future reports.

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Gas-Liquid Chromatographic-Thermal Energy Analyzer Determination of *N*-Nitrosodimethylamine in Beer at Low Parts per Billion Level

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A gas-liquid chromatographic (GLC) procedure is described for the determination of low ppb levels of *N*-nitrosodimethylamine (NDMA) in beer. The sample is treated with sulfamic acid under acidic conditions followed by alkalinization with dilute KOH, distillation at atmospheric pressure, extraction of NDMA from alkaline aqueous distillate with dichloromethane, concentration of dichloromethane extract by Kuderna-Danish concentrator, and, finally determination by a GLC-thermal energy analyzer technique with *N*-nitrosodipropylamine (NDPA) as an internal standard. The method gave results comparable with 2 other well established methods. Recoveries of added NDMA, *N*-nitrosodiethylamine, and NDPA at levels ranging from 0.08 to 10 ppb were 75-112, 86-115, and 85-109%, respectively. Five replicate analyses of a beer sample gave a mean NDMA concentration (corrected for recovery) of 2.21 ± 0.08 ppb (± SD). Minimum detection limit of the method is about 0.1 ppb.

Various methods have been used to determine trace levels of *N*-nitrosodimethylamine (NDMA) in beer and other alcoholic beverages. These include thin layer chromatography (1), gas-liquid chromatography-electron capture detection of the nitramine derivatives (2), GLC-high resolution mass spectrometry (3-5), and GLC-thermal energy analyzer (GLC-TEA) determination (3-7). The GLC-TEA technique appears to be the most sensitive, rugged, and trouble-free. Besides the many detection techniques, various laboratories have used different isolation and cleanup methods. Although end determinations are quite sensitive with most methods based on GLC-TEA (8), the accuracy and reproducibility of the overall procedures have not been tested adequately.

As a result of improved malt drying techniques (9), the concentration of NDMA in beer has been decreasing steadily during the last year. A reliable method is needed which can give fairly accurate results even at low ppb concentrations. Per capita consumption of beer and ale is quite high in many countries (3, 4); therefore, even at 0.1-1 ppb concentrations, these beverages contribute a major portion of the total daily intake of nitrosamines.

This paper describes a highly accurate and reproducible method which is sensitive to 0.1 ppb NDMA in beer and ale.

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The recommendation of the Associate Referee was approved by the General Referee and Committee C and was accepted by the Association. See *J. Assoc. Off. Anal. Chem.* **64**, 427 (1981).

METHOD

Caution. *N*-Nitrosamines are potent carcinogens; take adequate precaution to avoid exposure. Carry out all steps, wherever possible, in well ventilated fume hood and wear protective gloves while handling nitrosamine standards. Use mechanical pipetting aids for measuring all solutions. Use separate pipetting device for measuring standards and mark it appropriately; do not use it for pipetting other reagents. Because these compounds are highly photolabile, all work should be carried out under subdued light. Destroy all nitrosamine standards by boiling with HCl, KI, and sulfamic acid (10) before disposal.

Reagents

All chemicals should be analytical grade.

(**a**) *Dichloromethane (DCM).*—Distilled in glass. Test each bottle before use. Concentrate 200 mL to 1 mL as described under *Concentration* and then analyze 10 μL aliquot by GLC-TEA. Test must show absence of NDMA.

(**b**) *Sodium sulfate.*—Anhydrous (granular). Test each bottle as follows: Dissolve 40 g in 50 mL water, add 2 mL 10N KOH, extract with two 50 mL portions of DCM, dry extract over anhydrous Na_2SO_4 as described later, concentrate to 1 mL, and analyze 10 μL aliquot by GLC-TEA. Extract must be free of NDMA.

(**c**) *Distilled or deionized water.*—Test as follows: Take 50 mL water, add 2 mL 10N KOH, extract with two 50 mL portions of DCM, and test for NDMA contamination as under (**b**). If test under (**b**) is negative (which includes 50 mL water), there is no need to test water separately.

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(d) *Boiling aids.*—Boileezers® (Fisher Scientific Co., Catalog No. B-365), or equivalent.

(e) *Sulfamic acid.*—10% in water; store at 4°C.

(f) *KOH.*—10N and 1N; store in polyethylene bottle.

(g) *NDMA.*—(*1*) *Stock solution, 10 mg/mL.*—Accurately weigh (±0.0001 g) ca 100 mg in 10 mL volumetric flask (with polyethylene stopper), dilute to mark with DCM, and mix well. Store at −20°C, and warm to room temperature in the dark before use. Prepare fresh stock solution once a year. (*2*) *Dilute solutions.*—By serial dilutions (using ≥1 mL pipets) (*1*, above), prepare the following NDMA solutions in DCM: 500, 200, 100, 40, 20, 10, and 5 ng/mL. Store at −20°C and warm to room temperature in the dark before use. Prepare fresh dilute standards once a month.

(h) *N-Nitrosodi-n-propylamine (NDPA) standards.*—As described above, weigh and prepare solution containing 250 ng NDPA/mL anhydrous ethanol. Note: Diluted standards available from commercial firms are acceptable. Always use appropriate solvents (DCM for NDMA and ethanol for NDPA) for subsequent dilutions.

Apparatus

(a) *Graham condenser.*—No substitutes, with $^{24}/_{40}$ joints, jacket length 200 mm (Kontes No. K-439000).

(b) *Kuderna-Danish (K-D) evaporative concentrator.*—250 mL capacity, with $^{24}/_{40}$ column connection and $^{19}/_{22}$ lower joint, complete with springs (K-570000).

(c) *K-D Concentrator tube.*—4 mL capacity, with $^{19}/_{22}$ joint, and 0.1 mL subdivisions from 0 to 2.0 mL (Kontes No. K-570050). Check accuracy of graduations. Use with pennyhead stoppers ($^{19}/_{22}$ joint).

(d) *Snyder column.*—3 section, 150 mm, with $^{24}/_{40}$ joints (Kontes No. K-503000).

(e) *Micro Snyder column.*—3 chambers, with $^{19}/_{22}$ joint (Kontes No. 569001:3-19).

(f) *GLC column.*—6 ft × $^1/_8$ in. (od) stainless steel column packed with 20% Carbowax 20M and 2% NaOH on 80–100 mesh acid-washed Chromosorb P. Column must be able to handle 10 μL sample extract and must give good resolution of NDMA peak from both solvent (in beer extract) and NDPA peaks. Injector and column temperatures, 220°C and 170°C, respectively. Carrier gas (Ar) flow, 25–30 mL/min.

(g) *GLC-thermal energy analyzer.*—Thermo Electron Corp., Waltham, MA, connected to 1 mV recorder. Operate according to instrument manual and with −110°C to −130°C slush bath. Adjust instrumental parameters, such as vacuum chamber pressure, oxygen flow, calibration knob, etc. to obtain proper sensitivity. Set recorder chart speed at ca 0.5 cm/min.

Note: Thoroughly clean all glassware before use. After normal cleaning and washing, wash with chromic acid. If contamination still exists, rinse all glassware with DCM before use. Let charred residue in distillation flask soak with dilute alkali and then wash in normal manner.

Sampling and Storage

Store beer sample at 4°C in dark and analyze as soon as possible. When opening bottle or can, transfer ca 120 mL aliquot into glass-stopper Erlenmeyer flask and store as above. Alternatively, recap bottle, using bottle capper, after taking aliquot. In the latter case, test 4% ethanol extract of new cap liner before use for NDMA contamination.

Distillation

Accurately weigh 50 ± 0.1 g beer into 1 L round-bottom distillation flask and add 1.0 mL each of 10% sulfamic acid, NDPA internal standard (250 ng/mL), and 1N HCl. Mix contents by gentle swirling and let stand in dark 10 min. Then add 10.0 mL 1N KOH and 2 small Boileezers, and mix. Set up distillation apparatus so that connecting adapter slopes downward toward vertical Graham condenser. Loosely wrap glass wool around distillation flask and connecting adapter. Set up 250 mL separatory funnel under condenser to collect distillate. Cooling water for condenser should be ≤20°C.

During initial 10 min of distillation, adjust rheostat (usually at 50) so that mixture boils smoothly without too much frothing or bumping. Watch constantly for excessive foaming and, if necessary, turn off heat for 1–2 min. After 10 min, increase rheostat setting to 60 or 65 and continue distillation (watch for foaming) until most of solution is distilled. Stop distillation when ca 2–3 mL liquid remains in distillation flask. *Do not heat to complete dryness*; this may give erroneous results. Total distillation time should be ≤1 h. If any portion of sample foams over during distillation, discard experiment and start over with fresh aliquot.

Extraction and Cleanup

Disconnect adapter after distillation; do not rinse adapter. Add 2.0 mL 10N KOH to distillate, rinse condenser with 50 mL DCM, and col-

lect rinsing directly into separatory funnel containing distillate and KOH. Extract distillate with DCM by shaking vigorously 2 min and drain off DCM layer into second separatory funnel. Extract aqueous layer with 2 additional 50 mL portions of DCM and combine all DCM extracts in second separatory funnel. Discard aqueous layer.

Place 40 g anhydrous Na_2SO_4 in coarse sintered-glass Buchner funnel, wash with ca 20 mL DCM, and discard washing. Assemble 250 mL K-D evaporative concentrator with 4 mL concentrator tube at bottom. While connecting bottom tube, wet joint with DCM and attach springs. Dry combined DCM extract by passing through Na_2SO_4 bed on Buchner funnel and collecting extract directly in K-D concentrator. Wash Na_2SO_4 bed with further 20 mL DCM and collect washing in K-D concentrator.

Concentration

Add 1 tiny piece of Boileezer to contents of K-D flask, attach 3-section Snyder column, and concentrate extract by heating flask in water bath (50–60°C). Initially maintain outside water level close to level of DCM inside flask and continue heating until concentrated extract is ca 4 mL (ca 40 min). (If excessive boiling occurs during concentration, control it either by raising flask slightly out of water bath or by decreasing temperature of bath.) Finally raise flask above water and allow condensed DCM in Snyder column to drain into flask. Add ca 1 mL DCM to top of Snyder column and let it drain to flask. Disconnect concentrator tube from flask.

Add another tiny piece of Boileezer to contents and attach micro Snyder column and springs. Concentrate extract to ca 0.8 mL by heating concentrator tube in 50–60°C water bath. Lift out or immerse tube in water to control boiling rate but *do not* lift tube completely out of water bath; this will stop action of Boileezer. *Avoid overheating and excessive accumulation of DCM in column chambers.* Stop concentration when DCM level reaches ca 0.8 mL; do not concentrate to less than 0.8 mL. Carry out this final concentration step slowly, taking at least 30 min. Raise tube (bottom still touching water), let liquid drain, and note volume to see if it is ca 0.8 mL. If >0.8 mL, continue concentration as above. Finally, rinse micro Snyder column with a few drops of DCM, let rinsing drain to tube, disconnect column, and dilute extract to 1.0 or 1.1 mL (not >1.1 mL). (Do not use nitrogen stream for concentrating extract at any stage.)

Stopper tube, mix in Vortex mixer, and store at 4°C in dark until analysis. Let warm to room temperature and note volume before analyzing extract.

Reagent Blank

To ensure absence of contamination, carry out reagent blank taken through all the steps as mentioned above, except use 50 mL 4% ethanol in water instead of 50 g beer. Inject 10 μL extract for GLC-TEA analysis as described below.

Determination of Standard Curve

Set attenuation (usually 4) of TEA detector so that injection of 30 pg NDMA gives definite peak with acceptable background. Using this attenuation, analyze 6 μL aliquots, in duplicate, of NDMA standards 5, 10, 20, and 40 ng/mL. Before injection draw out syringe plunger slightly and note exact volume of extract to be injected (there must be a small air gap between sample and rinsing solvent already inside needle). During injection make sure no sample is lost through back of plunger due to back pressure. After injection hold needle in septum 5 s before withdrawing.

Next, choose a higher attenuation setting that gives on-scale peak for 6 μL of NDMA standard 500 ng/mL. Using this setting, analyze 6 μL aliquots, in duplicate, of NDMA standards 500, 200, 100, and 40 ng/mL.

Accurately measure peak heights (±0.1 cm) and determine average peak heights of 2 injections at each concentration. If exactly 6 μL is not injected, make appropriate corrections and convert all peak heights equivalent to 6.0 μL injections. Draw 2 standard curves, one for each attenuation setting, peak height vs. pg injected. Determine standard curve weekly.

Analysis of Beer Extract

As above, inject 6 μL aliquots of beer extract, in duplicate, using lowest attenuation setting sensitive to 30 pg NDMA. Measure and determine average peak height corresponding to 6.0 μL injection. Compare this average peak height with standard curve and determine which standard NDMA solution, when injected under same attenuation setting, produces closest peak height. Choose that NDMA standard solution, inject 6 μL aliquots, in duplicate, and determine average peak height.

If sample extract on first injection produces off-scale peak, choose a higher attenuation set-

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Table 1. Comparison of results (NDMA levels in ppb) obtained by 3 methods for determining NDMA in beer

Sample	This method			Method 1 (4)		Method 2 (6)		
	Uncorrected, ppb	Corrected,[a] ppb	Recovery, %	Uncorrected, ppb	Corrected,[b] ppb	Uncorrected, ppb	Corrected,[a] ppb	Recovery, %
Dark beer, Brand A	6.7	7.4	90.7	5.9	7.4	—	—	—
Beer, Brand B[c]	4.9	5.8	84.7	—	—	4.7	5.7	82.4
Porter, Brand C	0.8	0.9	88.8	0.7	0.9	—	—	—
Malt liquor	0.33	0.4	88.0	0.4	0.5	0.3	0.4	79.0
Beer, Brand B[c]	9.7	9.7	102.0	—	—	8.6	9.7	88.6
German beer	2.3	2.3	102.0	2.1	2.6	2.1	2.5	83.3
Light beer, Brand D	0.72	0.8	90.3	0.64	0.8	—	—	—
English beer, Extra stout	0.96	1.0	91.3	0.83	1.0	—	—	—

[a] Corrected for % recovery of NDPA.

[b] Since Method 1 (4) uses a vacuum distillation technique, the average percent recovery of NDMA, which is more volatile, is lower than that of NDPA. For this reason, all results are corrected for an average (of 5 determinations) of 80% NDMA recovery instead of correcting against NDPA recoveries.

[c] Beer samples spiked with known levels of NDMA.

ting (16 or 32) and carry out analysis, in duplicate, as above. Also analyze corresponding standard NDMA solution at same attenuation. For samples giving off-scale peaks at attenuation 32, dilute extracts with DCM to 5.0 mL in a volumetric flask and re-analyze. *For accurate results, analyze beer extract and corresponding standard under same attenuation setting and all within 60 min.*

If, on the other hand, extract gives negative result for NDMA or peak is too small to measure, inject 10 μL aliquots, in duplicate (use 25 μL syringe). Similarly, inject duplicate 10 μL aliquots of NDMA standard 5 ng/mL for quantitation. To achieve 0.1 ppb detection limit, 10 μL aliquots of beer extract must be analyzed under attenuation setting that gives detectable peak for 30 pg NDMA.

Note: If using 25 μL syringe, which usually has thick needle, watch for septum damage and check for leaks. To be on safe side, *use a new septum* daily.

Calculation

Calculate concentration of NDMA in beer, using the following formula:

Uncorrected ppb NDMA in beer = $(h_1 p v_2)/(h_2 g v_1)$, where h_1 = average NDMA peak height (cm) of beer; h_2 = average peak height (cm) of corresponding NDMA standard; p = pg NDMA that produced h_2 peak height; v_1 = μL beer extract injected; v_2 = final volume (mL) of beer extract; g = g beer taken for analysis.

Correction for % recovery of NDPA.—Accurately measure peak height of NDPA peak on each beer chromatogram and calculate average peak height of 2 injections. Make appropriate corrections if final volume of beer extract is not exactly 1.0 mL or injection volume is not exactly 6.0 μL. Then (within 60 min) inject, in duplicate, 6 μL NDPA standard (250 ng/mL) under same attenuation setting. Calculate average peak height and correct value if exactly 6.0 μL is not injected. Calculate % recovery of added NDPA for each sample. If recovery of NDPA is less than 80%, repeat analysis from beginning. Finally, correct results as follows:

Corrected ppb NDMA in beer = (uncorrected ppb/% recovery of NDPA) × 100.

Results and Discussion

In previous studies in this laboratory, both atmospheric distillation from KOH solution (1) and vacuum distillation from KOH or sulfamic acid solutions (4) have been used for the determination of volatile nitrosamines in alcoholic beverages. Because of its simplicity and slightly better reproducibility, the atmospheric distillation technique was chosen in the present study. The method is somewhat similar to the barium hydroxide method of Fazio et al. (6), but the 2 methods differ in several ways. For example, a 10 min incubation of the sample with acidic sulfamic acid has been introduced to destroy any nitrosating agent that might be present. The

Table 2. Percent recoveries [a] of various nitrosamines added to beer

NDMA		NDEA		NDPA	
Added, ppb	Rec., %	Added, ppb	Rec., %	Added, ppb	Rec., %
4	85.9	4	85.6	4	86.7
4	88.0	4	89.8	4	87.9
4	91.8	4	88.3	4	91.2
4	89.5	4	94.9	4	90.8
5.7	86.0	—	—	5	84.7
9.3	104.0	—	—	5	102.0
0.8	90.7	0.8	89.3	0.8	92.9
0.16	112.0	0.16	115.0	0.16	109.0
0.08	75.0	—	—		—

[a] After subtracting amount present in unspiked beer.

distillation condition and the final concentration technique are also more efficient and rugged than those previously reported (6). Addition of an extra 10 mL water to sample as 10 mL 1N KOH just before distillation in this method ensures good recoveries for most of the volatile nitrosamines. Because of higher sample size and larger aliquots injected, the overall sensitivity (see below) of the method is much better than 0.5 ppb reported by Fazio et al. (6). Although the method may be more time consuming than some rapid screening methods (e.g., ref. 5), it gives more precise and accurate results at low levels. With 2 distillation set-ups, an experienced technician can run 4 samples per day.

The method has been tried with several samples of beer and the results have been compared with those obtained with a vacuum distillation method (4) as well as with the method of Fazio et al. (6). As can be seen from the data in Table 1, agreement is excellent. The percentage recoveries (Table 2) of NDMA added to various beer samples were also good at all levels and were not significantly different from those of NDPA (internal standard). The minimum detection limit of the method is 0.1 ppb NDMA. Typical GLC-TEA chromatograms obtained by this method are shown in Figure 1.

To test the ruggedness of the method, some of the conditions were changed as follows: (a) carrying out the entire experiment (including incubation with sulfamic acid) under normal laboratory light (fluorescent lamps), (b) using water at 20°C, instead of normal cold water from tap, for cooling condenser, (c) concentrating the final extract to as low as 0.4 mL, and (d) using 2 mL 3N KOH (instead of 10N) for basifying the distillate. The first 3 changes did not result in detectable loss of added NDMA, whereas the last change gave a slightly lower recovery for NDMA. We suggest that the analysis be carried out under subdued light as described in the method.

An attempt was also made to determine the amount of NDMA left in the aqueous distillate after 3 extractions with DCM. This was done by dissolving 4 g KOH in the aqueous distillate left behind after 3 extractions with DCM, and extracting it once more with 50 mL DCM. The amount of NDMA recovered in the fourth extract

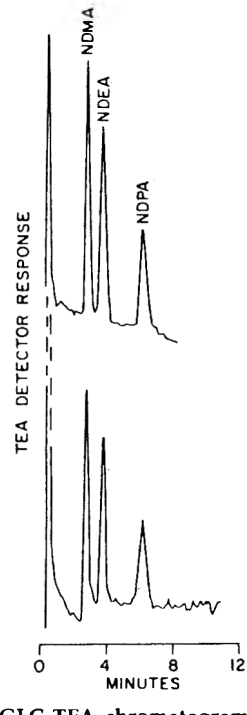

Figure 1. GLC-TEA chromatograms: Lower – 5.1 μL/1.0 mL final extract of beer spiked with 0.8 ppb each of NDMA, *N*-nitrosodiethylamine (NDEA), NDPA; attenuation setting 4 (unspiked beer gave no detectable peak). Upper – 5.1 μL standard solution containing 204 pg of each nitrosamine.

Table 3. Stability of NDMA in beer after storage at 30°C

Sample	NDMA level (ppb) found by analysis: Before storage	After storage[a]
Brand D (light beer)	0.72	0.75
German beer	2.30	2.20
Negative beer spiked with 10 ppb NDMA	9.70	8.70

[a] Two weeks in the dark at 30°C.

was negligible (0.9% of total); therefore, a fourth extraction with DCM would not significantly increase the efficiency of the technique.

During transport, beer samples may be exposed for short durations to warm temperatures. To determine if such exposures could adversely affect NDMA levels in beer, the following experiment was carried out: Three bottles of beer, each containing a different concentration of NDMA, were analyzed, recapped, wrapped in aluminum foil, and stored at 30°C. After 2 weeks, the samples were reanalyzed. Two samples (Table 3) showed no detectable changes, but a slight loss of NDMA was observed from the 3rd sample.

Finally, the reproducibility of the entire procedure was tested by replicate analyses of a beer sample. As shown in Table 4 the precision for both the uncorrected and corrected values is excellent. Similarly, triplicate analyses of another commercial beer gave uncorrected NDMA levels of 0.57 ppb, 0.52 ppb, and 0.57 ppb (analyses done on different days) with corresponding NDPA recoveries of 103.8%, 96.8%, and 97.3%, respectively. All the analyses were done by the same analyst.

In conclusion, the method is very simple and straightforward and does not involve the use of any elaborate distillation set-up. It is also very sensitive and reproducible, and it gives results comparable to other published methods.

Note added in proof: It has since been found that adding 10 mL 3N KOH instead of 10 mL 1N KOH results in less foaming during distillation of some beer samples. This change does not alter recoveries or reproducibility.

Table 4. Precision of the method as determined by 5 replicate analyses from a bottle of Dutch (imported) beer

Number of determination	NDMA level (ppb): Uncorrected	Corrected for % Rec. of NDPA	Rec. of added NDPA, %
1	2.14	2.15	99.5
2	2.19	2.21	98.9
3	2.26	2.35	96.2
4	2.08	2.17	95.9
5	2.09	2.17	96.2
Mean ± SD	2.15 ± 0.07	2.21 ± 0.08	97.3 ± 1.72

Recommendation

It is recommended that the method be further studied to test precision and accuracy at various concentrations, i.e., limit of detection and twice limit of detection, to determine day-to-day intralaboratory variation. Then the method can be subjected to collaborative study.

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MYCOTOXINS

Evaluation of Laboratory Performance with Aflatoxin Methods by Means of the AOCS Smalley Check Sample Program

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The American Oil Chemists' Society Smalley Check Sample Program offers ongoing check sample series for determination of aflatoxins in peanut meal, cottonseed meal, and corn meal. Laboratories participating in this program represent a worldwide cross section of industry, regulatory, and commercial laboratories. Each annual series presently consists of 7 samples including a solution of an unknown mixture of aflatoxins B_1, B_2, G_1, and G_2 for direct spotting and quantitation. Participant analyses of the solution of aflatoxins resulted in coefficients of variation generally higher than those of any sample. Comparison of 4 years of results for analysts using BF and CB methods of analysis for peanut meal samples with aflatoxin B_1 levels ranging from 2 to 56 ng/g resulted in higher aflatoxin B_1 results for the CB method. The number of participants reporting results by HPLC methods is small; however, their results compare closely with those using TLC methods.

The Smalley Check Sample Program is a nonprofit service of the American Oil Chemists' Society, supported by a minimal subscription fee. Individual samples of aflatoxins in peanut meal, aflatoxins in cottonseed meal, aflatoxins in corn meal, and, beginning this year, aflatoxin M_1 in milk are available for participation by all interested analysts. Collaborators are identified by a random analyst number known only to the analyst and the few individuals involved in data handling and subscription.

The primary objective of the program is to provide laboratories who perform aflatoxin analysis an ongoing means of judging their analytical proficiency in using official AOAC, AOCS paraphrase, or European aflatoxin analytical methods; methods used are not specified, but they must be official methods and participants must record methods and techniques used in obtaining their sample results. In addition, a competitive stimulus exists in the program. At the completion of each annual series, certificates of outstanding proficiency are awarded to the highest ranking analysts. Cumulative results of the Smalley Program are used by the Examination Board of AOCS to annually certify analysts in commercial laboratories as "Referee Chemists"; the analyst must show continuing very high proficiencies to maintain this certification.

Accumulated analytical results of the broad cross section of worldwide industrial, regulatory, and commercial laboratories participating in the Smalley Series provide an opportunity to evaluate laboratory performance with aflatoxin methods in the plethora of environments, reagent qualities, standards, techniques, and analyst capabilities that may deviate considerably from collaborative studies.

Program Procedure

For the initial 2 seasons, the Smalley Program offered check sample series for aflatoxins in peanut meal, cottonseed meal, and, beginning in 1976–77, corn meal. Each consisted of a total of 6 season samples. Presently, all series consist of 7 season samples. Individual, carefully ground and blended, 100 g samples are mailed to participants for analysis at monthly intervals. In each series, Samples 1 and 3 are specified as duplicates; Sample 2 in each series is a sealed ampoule of a benzene–acetonitrile (98 + 2) solution of the 4 aflatoxins B_1, B_2, G_1, and G_2 for direct spotting and quantitation. Study of comparative results of the aflatoxin solution provides information on quality of standards and techniques that can be applied to subsequent samples. Unknown to participants, 2 of the remaining season samples in all series are duplicates. Results of these samples provide valuable insight into within- and between-laboratory analyst performance.

Participant analytical results are edited so that any determinations by unofficial or improper methods are excluded from evaluation, and aggregate accepted results are processed to compute arithmetic mean and estimates of standard deviation; all results outside ±3 standard deviations of the mean are excluded from evaluation. In addition, deviations relative to standard deviation or bias are calculated for each participant according to the formula: $t_{ix} = (x_i - \bar{x})/s_x$ for

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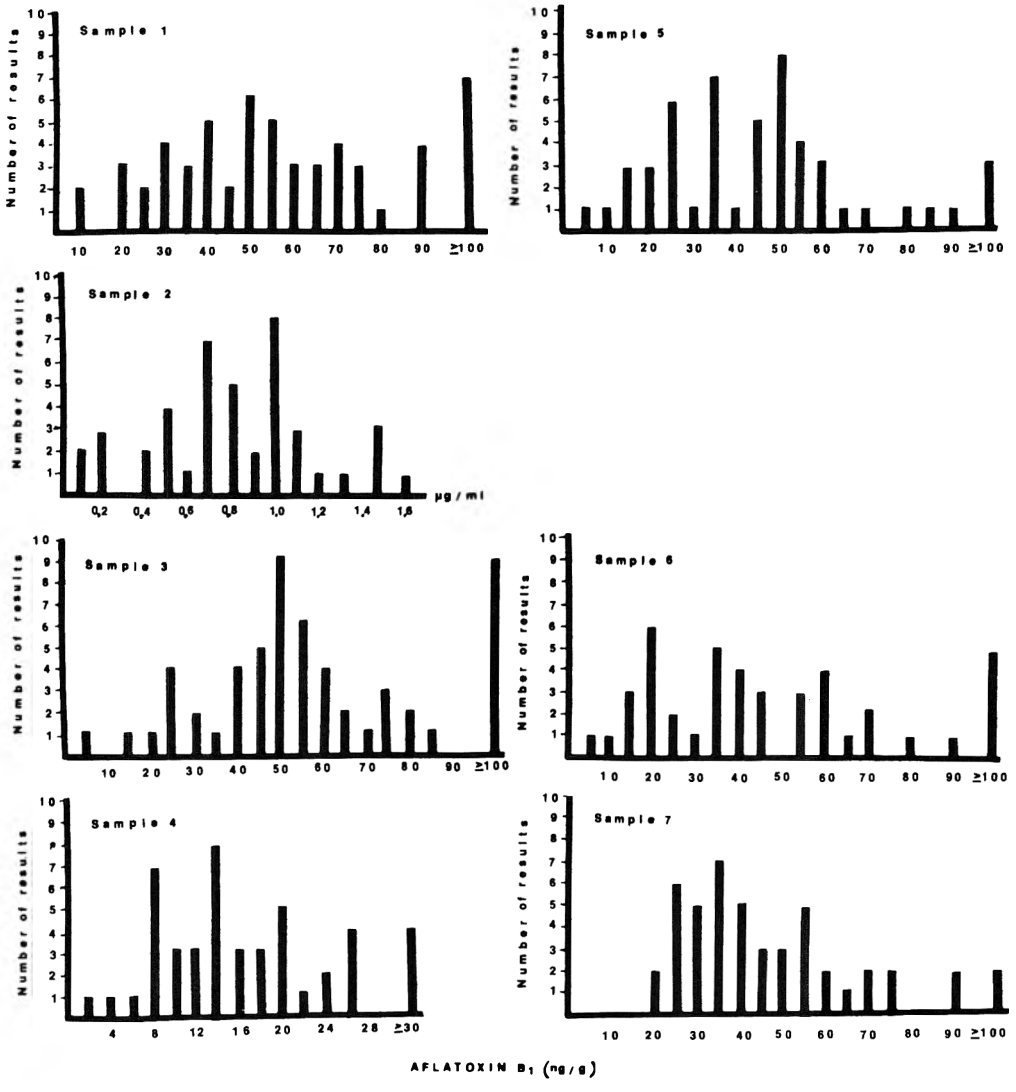

Figure 1. Results for aflatoxin B_1 in peanut meal.

individual and total aflatoxin determinations. Cumulative analyst results for total aflatoxin are used as a basis for ranking participants by proficiency index. This cumulative proficiency index is calculated according to the formula: $P_i = (\Sigma(t_{ix})^2/n)^{1/2}$, where t_{ix} = bias for individual and total aflatoxin results, x_i = analyst result, $\bar{x}$ = mean value for aflatoxin result, s_x = standard deviation for aflatoxin result, n = number of aflatoxin results, and P_i = cumulative proficiency index. The proficiency index for each participant is thus a measure of the difference of his/her results from the arithmetic mean of all results, expressed as a fraction of the standard deviation of all results. Proficiency indices are then ranked in inverse order of magnitude for recognition of analyst proficiency.

Results and Discussion

Frequency distribution curves for all aflatoxin B_1 results reported by those participants using accepted official aflatoxin methods for the 1979–80 season are presented in Figures 1–3 for peanut meal, cottonseed meal, and corn meal, respectively. All aflatoxin B_1 values were rounded to the nearest 5 ng/g, except those samples with a mean value less than 15 ng/g were rounded to the nearest 2 ng/g. Results for

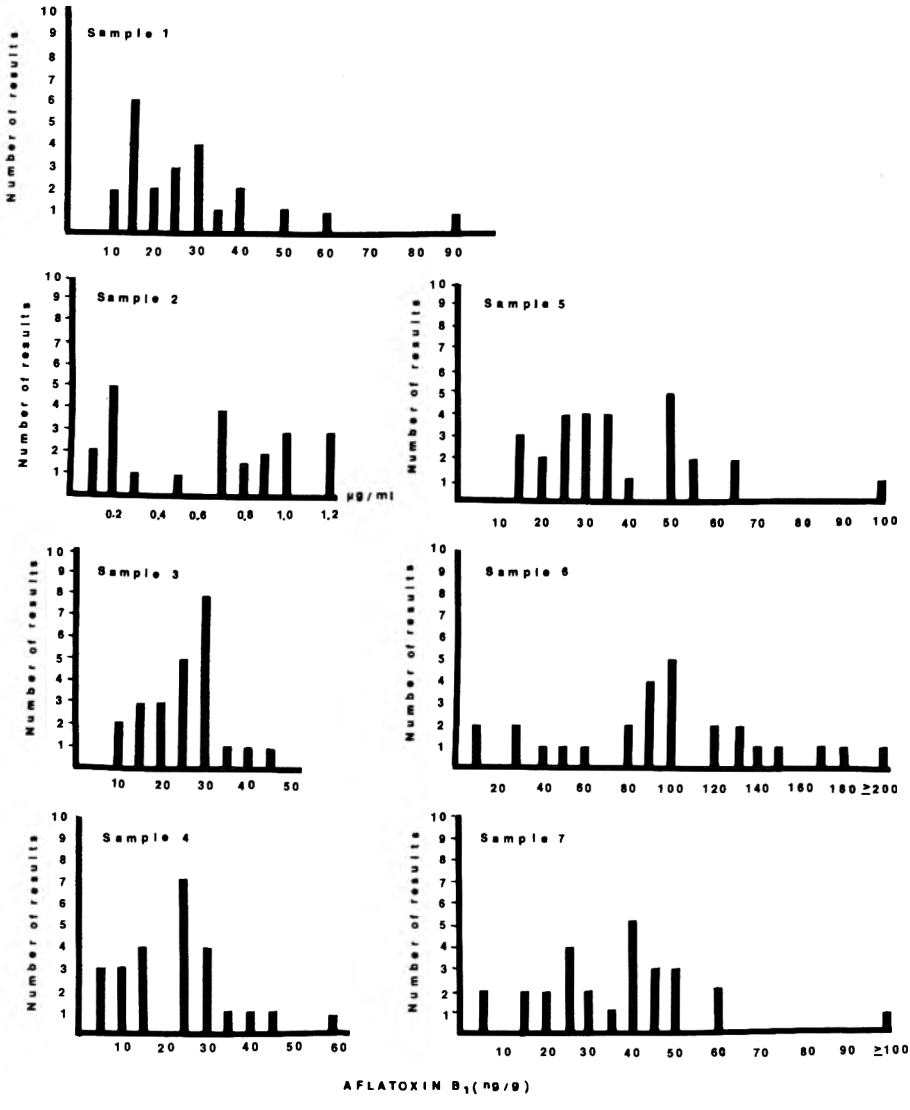

Figure 2. Results for aflatoxin B_1 in cottonseed meal.

Sample 2 in all series were rounded to the nearest 0.1 μg/mL. Inspection of results for all series shows little indication of distribution other than Gaussian, although many results, including those for the aflatoxin working solution, show a very wide distribution.

Evaluation of 5-season results for peanut and cottonseed meals and 4-season results for corn meal are presented in Tables 1–3, respectively. These tables illustrate the continuing high coefficients of variation in all series, although 1979–80 season results for the peanut meal series show considerable improvement in precision of results over past seasons, and those for cottonseed meal and corn meal are improved somewhat. Participant analysis of Sample 2 (unknown working solution of aflatoxins) for the past 2 seasons resulted in coefficients of variation that were generally higher than those of any sample. These results again support the view that quality of standards and the TLC phase of aflatoxin analysis are major contributors to variation in analytical results.

Table 4 presents a 4-season evaluation of aflatoxin B_1 results in peanut meal for analysts using BF and CB methods, where analysts using each method are different. These results show a higher mean value for most but not all samples

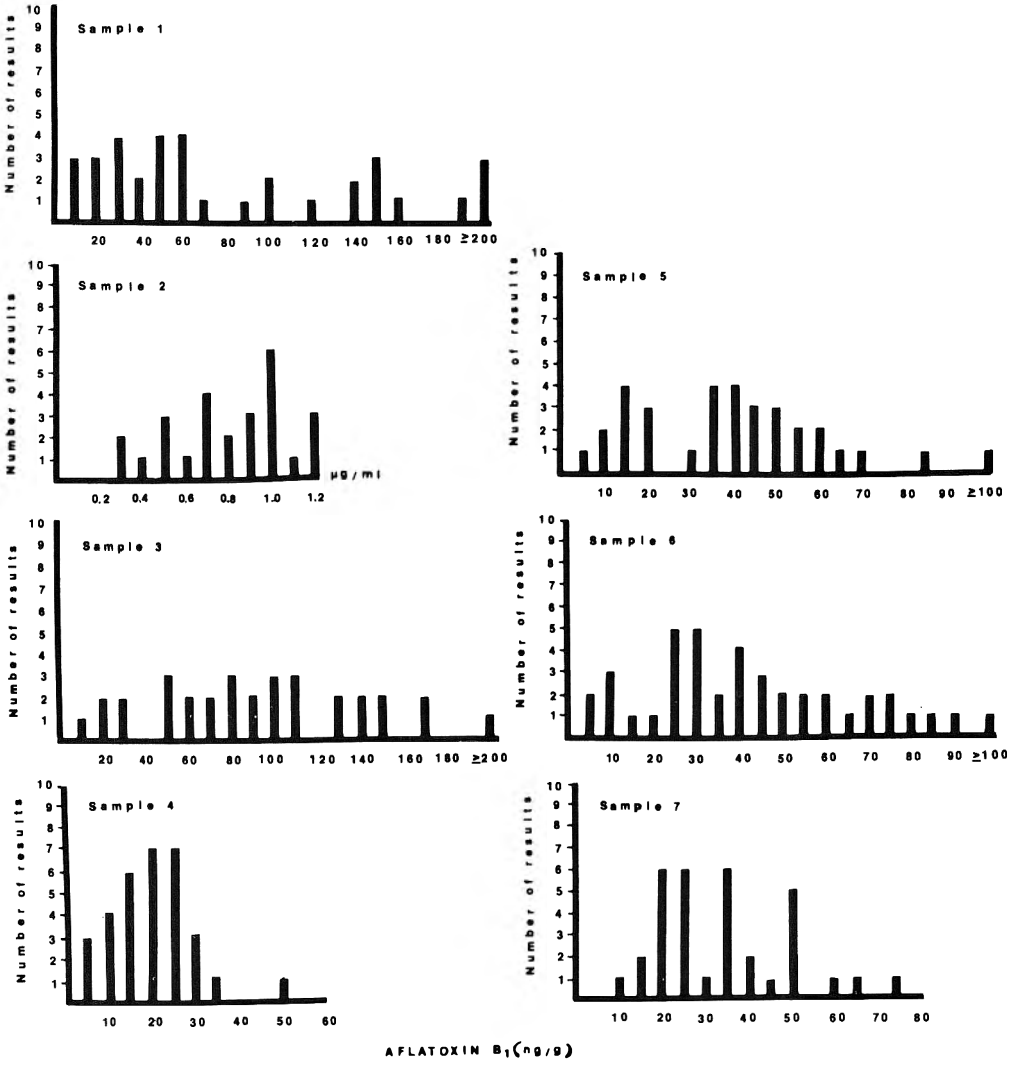

Figure 3. Results for aflatoxin B_1 in corn meal.

for those analysts using the CB method. Fewer participants in this series report using the CB method, with a consequent decrease in degrees of freedom for evaluation of results by this method. A study of the 2-method evaluation indicates a distinction in comparative results: Those samples containing greater than 50 ng/g total aflatoxin averaged 38.3% higher aflatoxin B_1 values for the CB method; those samples containing less than 50 ng/g total aflatoxin averaged 18.9% higher aflatoxin B_1 values. These continuing differences, compared with the collaborative results of Waltking (1) and Stack (2), where methods were found to be equivalent in accuracy and precision within this range of aflatoxin levels, indicate that many analysts are continuing to use faulty techniques, not following certain aspects of procedures, or persisting in other errors that cumulatively result in these differences. However, these results and those of the 1980 International Mycotoxin Check Sample Program (3) indicate that analyses by the 2 methods of samples containing low levels of aflatoxin agree closely.

Table 5 presents a 2-season evaluation of laboratory performance with duplicate samples unknown to participants. Results of analysis for aflatoxin B_1 for the 3 series were evaluated for

Table 1. Evaluation of participant analyses of aflatoxins in peanut meal series

Sample	N[a]	Mean, ng/g					Std dev., ng/g					Coeff. of var., %				
		B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2	Total
1975-1976 Season																
1	22	19	4	15	3	38	16	4	12	3	32	86	119	62	85	80
2[b]	28	13	3	12	3	31	8	2	9	2	17	56	82	78	77	54
3	29	22	5	21	6	53	12	4	14	4	30	53	73	67	78	56
4[b]	27	13	3	12	3	34	7	3	7	2	20	57	80	59	70	58
5	25	10	2	5	1	20	6	2	5	1	13	55	89	111	98	63
6	30	9	2	4	1	23	7	2	6	1	21	77	111	133	144	107
1976-1977 Season																
1	23	15	2	9	1	31	15	2	8	1	28	99	96	88	118	91
2	25	26	5	21	4	57	14	3	14	3	29	54	65	67	68	52
3	28	31	6	16	4	57	18	5	12	3	29	56	84	72	84	52
4[b]	30	25	5	12	3	48	13	4	6	2	24	53	75	55	92	50
5	36	21	4	14	3	45	12	3	7	2	22	56	71	48	67	49
6[b]	32	26	5	16	3	50	14	4	9	3	25	53	80	56	85	50
1977-1978 Season																
1	48	13	3	8	2	27	8	1	5	1	13	57	56	58	68	48
3	52	14	2	8	2	27	8	1	4	1	15	59	63	58	82	53
4	50	19	4	11	3	36	10	3	5	2	17	54	76	51	74	46
5[b]	50	16	3	10	2	32	8	2	6	2	15	50	59	60	70	45
6[b]	52	16	2	3	1	22	7	1	3	1	11	46	75	100	106	49
7	48	2	1	1	1	3	3	1	2	1	4	136	138	151	170	122
1978-1979 Season																
1	44	42	7	8	2	57	30	4	6	2	35	71	57	76	95	60
2[c]	35	0.36	0.15	0.13	0.31	0.98	0.30	0.13	0.13	0.27	0.80	83	84	102	85	81
3[b]	42	43	6	6	2	54	25	4	4	2	26	58	56	64	93	49
5[b]	43	43	7	8	2	53	31	5	8	2	31	70	70	99	117	60
6	41	50	9	9	3	72	31	5	8	2	40	62	62	82	81	55
7	40	35	7	7	2	52	20	4	5	2	26	57	58	69	91	50
1979-1980 Season																
1	55	55	7	25	5	114	32	4	14	3	83	59	50	57	58	73
2[c]	48	0.71	0.28	0.23	0.13	1.4	0.43	0.18	0.15	0.09	0.84	61	65	67	70	60
3	56	56	9	33	5	104	28	4	14	2	43	50	47	44	43	41
4	46	14	3	10	2	29	7	2	7	2	12	45	68	63	77	40
5[b]	55	41	6	23	4	76	20	4	12	3	32	49	56	52	71	42
6	47	45	7	20	5	77	26	4	9	3	35	58	53	48	72	45
7[b]	53	42	7	25	5	76	20	4	14	3	23	48	51	56	63	30

[a]Number of participant results.

[b]Duplicate samples, unknown to participants.

[c]Values for a working solution of the aflatoxins (µg/mL) for direct spotting.

Table 2. Evaluation of participant analyses of aflatoxins in cottonseed meal series

Sample	N[a]	Mean, ng/g					Std dev., ng/g					Coeff. of var., %				
		B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2	Total
1975-1976 Season																
1	16	20	6			30	10	5			21	53	86			72
2[b]	18	15	5			20	9	5			12	60	114			60
3	19	46	12			72	26	9			55	57	77			77
4[b]	19	15	4			19	8	3			10	55	86			53
5	18	26	6			32	12	4			14	44	67			45
6	17	27	8			40	17	8			25	63	96			63
1976-1977 Season																
1	16	8	2			13	5	1			9	66	64			70
2	12	18	4			22	12	4			14	68	84			63
3[b]	12	31	5			37	25	4			25	80	78			69
4	13	20	5			28	9	5			19	46	88			67
5[b]	16	27	6			35	12	4			17	45	61			48
6	14	15	3			20	10	4			15	68	129			77
1977-1978 Season																
1	17	17	5			23	7	2			8	42	44			37
3	17	16	4			20	7	2			7	43	46			33
4	19	29	7			40	12	3			13	41	45			34
5[b]	20	129	33			164	70	17			81	54	53			49
6	19	72	19			90	34	9			39	47	48			44
7[b]	20	116	31			175	71	15			136	61	49			78
1978-79 Season																
1	20	18	4			22	7	3			9	40	7			40
2[b]	17	0.41	0.14	0.19	0.28	1.0	0.35	0.12	0.16	0.23	0.76	84	81	85	82	73
3	20	17	4			21	5	2			6	28	45			30
4	19	25	7			32	8	5			12	32	67			37
5[b]	20	39	9			49	23	4			25	58	47			51
6	20	81	17			100	59	8			67	73	49			67
7[b]	19	38	9			48	13	4			17	35	41			36
1979-1980 Season																
1	23	24	5			34	13	3			21	53	57			62
2[b]	23	0.64	0.23	0.19	0.10	1.1	0.42	0.18	0.15	0.09	0.77	66	80	81	86	67
3	21	24	5			30	9	2			10	38	41			35
4	25	22	5			25	14	3			13	64	50			53
5[b]	25	37	8			45	21	4			26	58	54			58
6	26	93	20			113	53	12			64	57	61			56
7	27	33	8			41	16	8			20	49	47			49

[a]Number of participant results.

[b]Duplicate samples, unknown to participants.

[c]Values for a working solution of the aflatoxins (μg/mL) for direct spotting.

Table 3. Evaluation of participant analyses of aflatoxins in corn meal series

Sample	N[a]	Mean, ng/g					Std dev., ng/g					Coeff. of var., %				
		B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2	Total
1976-1977 Season																
1	16	10	2			12	6	2			6	59	81			49
2	16	19	5			24	8	3			9	42	57			40
3	16	7	2			10	4	1			4	55	74			44
4	19	10	3			15	6	3			8	56	98			51
5[b]	20	8	2			12	3	2			5	41	76			47
6[b]	19	8	1			11	3	1			4	31	88			41
1977-1978 Season																
1	24	20	5			25	10	4			13	53	73			52
3	23	18	5			24	8	2			8	46	50			35
4	24	12	3			16	7	2			6	61	63			41
5[b]	21	13	4			18	7	3			9	56	65			51
6[b]	25	14	4			18	7	3			8	48	63			44
7	24	48	5			53	34	4			36	70	82			68
1978-1979 Season																
1	32	17	2			21	12	2			11	68	123			54
2[c]	25	0.36	0.21	0.12	0.34	1.0	0.27	0.20	0.13	0.35	0.8	75	95	106	102	80
3	32	17	2			17	10	2			11	58	92			63
4	29	37	4			45	28	2			29	74	65			65
5[b]	30	34	3			39	23	3			24	68	81			61
6[b]	28	32	3			37	23	3			24	70	96			64
7	28	31	3			35	16	3			16	53	85			47
1979-1980 Season																
1	32	90	14			105	69	6			73	76	57			69
2[c]	28	0.74	0.31	0.24	0.14	1.5	0.34	0.16	0.12	0.07	0.61	46	52	51	50	40
3	32	92	12			104	49	7			54	53	56			52
4	29	17	1			22	8	1			10	49	91			44
5[b]	30	37	2			40	20	2			20	54	81			50
6	35	42	3			46	26	3			28	61	94			61
7[b]	33	32	3			35	17	2			17	53	81			49

[a] Number of participant results.

[b] Duplicate samples, unknown to participants.

[c] Values for a working solution of the aflatoxins (μg/mL) for direct spotting.

within-laboratory and between-laboratory precision according to the method of Youden (4). Unfortunately, many analyst results were eliminated from evaluation because of late reporting or failure to report results for one or both of the duplicate samples. Because the better analysts are traditionally the more dedicated ones, this evaluation is biased toward the results of these analysts; this bias is evidenced by coefficients of variation of between-laboratory results for this evaluation which are lower than those for the 2 individual duplicate samples in each series.

Table 4. Evaluation of participant analysis for aflatoxin B_1 (ng/g) in peanut meals by BF and CB methods

Sample	N[a]	BF method			CB method		
		Mean	Std dev.	Coeff. of var., %	Mean	Std dev.	Coeff. of var., %
1976-1977 Season							
1	15/6	13.3	14.2	106.0	21.7	19.7	90.6
2	16/8	20.6	11.4	55.4	40.3	9.5	23.7
3	17/8	27.5	17.1	62.0	45.1	8.8	19.5
4[b]	17/10	22.3	10.9	49.1	26.4	10.9	41.3
5	24/10	16.8	10.2	60.8	28.4	5.2	52.2
6[b]	20/10	18.1	10.0	55.3	31.6	12.5	39.6
1977-1978 Season							
1	25/19	12.2	6.7	55.0	14.5	8.1	56.0
3	30/15	12.7	6.9	54.3	15.1	8.9	58.9
4	34/12	15.8	8.0	50.6	25.1	12.9	51.3
5[b]	33/13	16.5	6.7	40.6	18.1	10.2	56.2
6[b]	32/16	16.7	6.7	40.0	14.6	7.6	52.0
7	30/13	2.5	3.4	136.0	1.8	2.1	119.0
1978-1979 Season							
1	30/12	31.8	21.5	67.6	53.3	32.5	61.0
3[b]	24/12	36.2	19.8	54.8	57.2	29.0	50.1
5[b]	24/12	36.9	29.1	78.9	58.8	27.8	47.5
6	22/13	36.9	20.8	56.4	72.8	34.8	47.9
7	22/13	29.7	15.7	52.9	46.7	25.4	54.4
1979-1980 Season							
1	36/10	44.0	22.6	51.4	68.4	34.5	50.4
3	31/10	47.2	20.2	42.8	73.4	37.3	50.8
4	26/10	13.5	5.6	41.2	16.8	9.3	55.5
5[b]	33/14	35.9	14.3	39.8	52.5	24.8	47.2
6	26/13	44.4	26.2	58.9	42.5	24.3	57.2
7[b]	27/12	40.5	17.0	42.0	46.3	24.9	53.7

[a] First number denotes number of participant analyses for BF method; second number denotes number of participant analyses for CB method.

[b] Duplicate samples, unknown to participants.

Both S_r, a measure of within-laboratory precision, and S_b, a measure of between-laboratory precision, have shown improvement in the 1979–80 season; in both seasons, between-laboratory error dominates in the results of all series.

Table 6 tabulates the number of results for all series outside 3, 2, and 1 standard deviation of the mean for 5-season results of all series. For the 1979–80 season, the average number of aflatoxin B_1 results outside 3 standard deviations of the mean was 1.6%, outside 2 standard deviations was 6.0%, and outside 1 standard deviation was 31.6%.

For the 1979–80 season, the mean value of annual proficiency indices or analysts' bias based on total aflatoxin value shows considerable improvement in precision of results for the peanut series compared with past seasons; those for cottonseed meal and corn meal were comparable to those of past seasons. These mean proficiency

Table 5. Two-season evaluation of laboratory performance with duplicate samples

	Peanut meal		Cottonseed meal		Corn meal	
	1978-1979 season	1979-1980 season	1978-1979 season	1979-1980 season	1978-1979 season	1979-1980 season
N [a]	33	38	17	22	23	23
Mean [b]	44.9	39.7	38.2	34.4	32.3	41.1
Std dev.	27.2	13.7	15.1	13.4	21.4	18.8
S_r	15.23	4.38	8.13	5.36	10.17	7.29
S_b	29.19	11.98	11.19	11.58	17.58	16.29
Coeff. of var. within-labs, %	33.9	11.0	30.1	15.6	31.4	17.7
Coeff. of var. between-labs, %	65.0	30.2	31.4	33.7	54.4	39.6

[a] Number of participants who ran duplicate determinations.

[b] Average of duplicate determinations.

Table 6. Number of results for all series outside 3, 2, and 1 standard deviations

Sample	Peanut meal						Cottonseed meal						Corn meal					
	Total N	N[a] outside	B_1	B_2	G_1	G_2	Total N	N[a] outside	B_1	B_2	G_1	G_2	Total N	N[a] outside	B_1	B_2	G_1	G_2
1975-1976 Season																		
		3	1	0	2	2		3	1	0								
1	22	2	2	2	3	3	16	2	1	1								
		1	5	4	7	9		1	8	3								
		3	0	0	0	0		3	0	0								
2	28	2	2	2	2	1	18	2	1	1								
		1	7	8	8	13		1	6	3								
		3	0	0	0	0		3	1	1								
3	29	2	1	1	2	2	19	2	2	2								
		1	11	10	9	7		1	5	5								
		3	0	1	1	1		3	0	0								
4	27	2	1	2	1	2	19	2	1	2								
		1	9	10	13	12		1	5	6								
		3	0	2	0	1		3	0	0								
5	25	2	0	3	1	2	18	2	1	0								
		1	8	10	4	15		1	5	7								
		3	2	2	0	3		3	0	1								
6	30	2	3	4	3	5	17	2	1	2								
1976-1977 Season																		
		3	0	1	1	2		3	1	3			3	0	0			
1	23	2	2	3	3	2	16	2	2	3			16	2	1	1		
		1	2	10	10	5		1	3	8				1	3	5		
		3	0	0	0	1		3	0	0			3	0	0			
2	25	2	0	0	1	1	12	2	0	1			16	2	0	0		
		1	9	11	6	11		1	4	2				1	5	6		
		3	0	0	0	0		3	0	0			3	0	0			
3	28	2	0	1	2	2	12	2	1	0			16	2	0	0		
		1	10	9	9	8		1	2	6				1	4	8		
		3	0	0	3	1		3	0	0				3	0	0		

Table 6. (cont'd)

Sample	Peanut meal						Cottonseed meal						Corn meal					
	Total N	N[a] outside	B_1	B_2	G_1	G_2	Total N	N[a] outside	B_1	B_2	G_1	G_2	Total N	N[a] outside	B_1	B_2	G_1	G_2
1976-1977 Season																		
4	30	2	1	1	3	3	13	2	0	1			19	2	0	1		
		1	9	8	13	10		1	6	3				1	7	6		
		3	1	1	2	2		3	0	0				3	0	0		
5	36	2	3	2	4	2	16	2	1	0			20	2	1	2		
		1	10	10	14	13		1	5	6				1	7	5		
		3	0	0	0	1		3	0	0				3	0	1		
6	32	2	2	2	0	2	14	2	1	1			19	2	0	1		
		1	9	8	10	13		1	4	1				1	6	7		
1977-1978 Season																		
		3	1	2	1	3		3	0	1				3	0	0		
1	48	2	2	2	2	6	17	2	0	1			24	2	0	1		
		1	16	18	19	15		1	7	6				1	6	8		
		3	1	2	4	1		3	0	0				3	0	0		
3	52	2	2	6	4	4	17	2	1	1			23	2	2	1		
		1	13	14	21	17		1	3	4				1	5	5		
		3	1	1	3	2		3	0	1				3	1	1		
4	50	2	4	5	4	5	19	2	2	1			24	2	2	1		
		1	17	13	14	17		1	3	9				1	6	10		
		3	1	0	0	1		3	0	0				3	0	0		
5	50	2	3	3	2	4	20	2	0	1			21	2	1	2		
		1	14	11	16	15		1	8	6				1	5	6		
		3	0	0	2	0		3	0	0				3	0	0		
6	52	2	3	4	3	1	19	2	0	0			25	2	1	1		
		1	15	12	10	8		1	5	8				1	6	8		
		3	1	1	1	0		3	1	0				3	0	0		
7	48	2	5	3	4	2	20	2	2	0			24	2	1	1		
		1	9	7	9	7		1	7	8				1	7	6		
1978-1979 Season																		
		3	1	1	1	3		3	1	1				3	0	2		
1	44	2	3	3	3	7	20	2	2	1			32	2	0	3		
		1	13	19	13	20		1	4	9				1	12	7		
		3	0	2	0	0		3	0	1	0	0		3	2	1	2	1
2	35	2	1	2	2	1	17	2	1	1	0	0	25	2	2	2	3	2
		1	18	24	5	17		1	5	8	4	5		1	14	9	5	5
		3	1	1	1	1		3	1	1				3	0	1		
3	42	2	2	1	2	4	20	2	2	2			32	2	2	2		
		1	14	11	15	17		1	6	5				1	8	16		
		3	0	1	2	0		3	1	0				3	0	2		
4	42	2	1	3	3	3	19	2	1	1			29	2	2	3		
		1	16	16	17	6		1	7	3				1	8	10		
		3	0	1	1	3		3	0	0				3	0	0		
5	43	2	3	4	4	5	20	2	2	1			30	2	0	1		
		1	9	13	15	10		1	5	5				1	13	13		
		3	1	0	0	1		3	0	1				3	0	1		
6	41	2	2	2	2	2	20	2	2	2			28	2	1	3		
		1	15	19	9	13		1	6	7				1	11	8		
		3	0	0	1	2		3	0	1				3	0	0		
7	40	2	2	3	3	4	19	2	1	2			28	2	1	2		
		1	10	14	12	16		1	5	6				1	8	9		

Table 6. (cont'd)

Sample	Peanut meal						Cottonseed meal						Corn meal					
	Total N	N[a] outside	B_1	B_2	G_1	G_2	Total N	N[a] outside	B_1	B_2	G_1	G_2	Total N	N[a] outside	B_1	B_2	G_1	G_2
1979-1980 Season																		
		3	2	3	5	2		3	1	1				3	0	0		
1	55	2	6	5	7	5	23	2	3	1			32	2	1	1		
		1	13	21	20	16		1	6	9				1	9	10		
		3	0	0	2	0		3	0	0	0	0		3	0	1	0	1
2	48	2	1	1	3	1	23	2	0	1	1	1	28	2	2	2	1	4
		1	16	18	20	21		1	10	8	8	11		1	8	7	10	6
		3	2	4	2	3		3	0	0				3	0	0		
3	56	2	5	8	4	6	21	2	1	2			32	2	0	2		
		1	16	21	18	17		1	6	8				1	8	9		
		3	4	3	0	3		3	0	0				3	0	3		
4	46	2	5	5	3	3	25	2	1	0			29	2	2	3		
		1	16	15	10	17		1	8	10				1	9	15		
		3	3	1	2	3		3	0	1				3	0	0		
5	55	2	6	4	5	6	25	2	1	1			30	2	1	0		
		1	16	21	23	15		1	7	8				1	12	16		
		3	2	1	3	1		3	0	0				3	1	1		
6	47	2	5	3	5	4	26	2	0	1			35	2	2	3		
		1	14	16	10	14		1	10	6				1	14	10		
		3	1	1	0	1		3	1	0				3	0	0		
7	53	2	5	4	2	4	27	2	3	2			33	2	1	1		
		1	15	15	13	14		1	6	8				1	13	8		

[a] Denotes number of standard deviations.

indices were: peanut meal 0.892; cottonseed meal 0.943; corn meal 0.931. The Smalley Committee Guideline states that proficiency indices less than 0.5 represent excellent-to-good analysis, 0.5 to 1.0 good-to-average analysis, 1.0 to 1.5 less than average-to-poor analysis, and 1.5 or greater as very poor analysis. Then 10.6% of participants reported excellent-to-good results, 54.1% reported good-to-average results, 28.1% reported less than average-to-poor results, and 7.3% reported poor-to-very poor results. These averages are for the results for all 3 series, because averages for individual series are very similar; the peanut meal series has a slightly higher percentage of participants doing good-to-excellent analysis.

No individual evaluation of HPLC results is presented because only an average of 4 participants in each series reported HPLC results; those results agreed closely with those of TLC, although slightly higher and with somewhat better correlation.

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High Performance Liquid Chromatographic Preparation of Alternariol, Alternariol Methyl Ether, and Altenuene

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A solvent system was developed for the isolation of 3 major *Alternaria* mycotoxins by high performance liquid chromatography (HPLC). Alternariol (AOH), alternariol methyl ether (AME), and altenuene (ALT) were purified using either a semipreparative or a preparative HPLC column. Gram quantities of pure mycotoxins were obtained in a single preparative HPLC step. Distribution of other individual mycotoxins and metabolites in various fractions obtained from either chromatographic procedure is discussed.

Although mycotoxin research has progressed rapidly in recent years, studies have concentrated on the toxins produced by the genera *Aspergillus*, *Penicillium*, and *Fusarium* (1). Limited emphasis has been placed on the toxic substances produced by fungi in the genus of *Alternaria*, which also commonly occur in foodstuffs and agricultural commodities (2–4).

Interest in the metabolites elaborated by *Alternaria* species originated from their antimicrobial activity and their potential role in the pathogenesis of certain plant diseases (5). The impact of these compounds on human and animal health was not immediately recognized until recent studies showed that many of the *Alternaria* species isolated from foodstuffs (2–4) and their respective metabolites are toxic to test animals (6, 7). As many as 7 metabolites are toxic in different test systems. Among these toxic metabolites, alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), and altenuisol (AS) are compounds containing a dibenzo-α-pyrone structure; tenuazonic acid (TA) is a tetramic acid, and the structures of 2 other toxins, namely, altertoxin I (AT-I) and altertoxin II (AT-II), have yet to be determined (6, 7). In view of the potential hazard of these toxins to human and animal health, further investigations are needed regarding their toxic effects and metabolic fate in animals, methods for toxin analysis, and stability in foods. Consequently, it is necessary to prepare a sufficient quantity of each toxin in pure form.

Although methods for isolating quantities of AME, AOH, and TA are available, most methods are time-consuming and involve solvent partitions (8, 9) or silica gel column chromatography (5, 10). Major *Alternaria* mycotoxins have been separated on the analytical HPLC column (11–13); therefore, we tested various solvent conditions for the preparation of these toxins by HPLC on semipreparative and preparative scales. Detailed methods for isolation of gram quantities of pure AME, AOH, and ALT are described below.

Experimental

Materials

(a) *Cultures.*—*Alternaria tenuis* and *A. alternata* used in the toxin production were obtained from R. D. Durbin, Department of Plant Pathology, University of Wisconsin-Madison, and from L. M. Seitz, Grain Marketing Research Laboratory, U.S. Department of Agriculture (USDA) (Manhattan, KS).

(b) *Standards.*—AME and AOH were supplied by D. J. Harvan, National Institute of Environmental Health Sciences (Research Triangle Park, NC). TA, ALT, AS, AT-I, and AT-II were obtained from R. M. Eppley, Food and Drug Administration (FDA) (Washington, DC).

(c) *Silica gel GF plates.*—Precoated (Analtech, Inc., Newark, DE).

(d) *Silica gel G-60.*—Scientific Products, McGaw Park, IL.

(e) *Columns.*—Two semipreparative silica gel HPLC columns, 7.8 mm id × 61 cm, packed with Porasil A and preparative, PrepPak-500 Silica Cartridge (Waters Associates, Milford, MA); analytical HPLC column, 4.6 mm id × 250 mm, Ultrasphere-ODS (Altex Scientific, Inc., Berkeley, CA).

(f) *Solvents.*—For analytical HPLC, all were HPLC grade (Burdick and Jackson) (Anspec Co., Inc., Ann Arbor, MI); for semipreparative and preparative HPLC, all were reagent grade (Fisher Scientific Co., Itasca, IL).

All other chemicals used in the study were either chemically pure or reagent grade.

Production of Alternaria Mycotoxins

All *Alternaria* cultures were maintained on potato-dextrose agar slants. Production of *Alternaria* mycotoxins was carried out in a rice me-

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dium inoculated with a spore suspension of either *A. tenuis* (A-2) or *A. alternata* (RL 8442-2). Cultures were grown in 2.8 L Fernbach flasks containing 300 g polished, long-grain rice obtained from a local grocery store and 150 mL tap water. Flasks were incubated 14 days at 25°C ± 1° in darkness. Cultures were shaken once a day by hand to break up the mycelial mat.

Preparation of the Alternaria Mycotoxin Extracts

After incubation, each rice culture was soaked overnight in 500 mL methanol, homogenized in an explosion-proof Waring blender for 1 min, and filtered under vacuum through No. 3 Whatman paper. The residue was homogenized with another 500 mL methanol and filtered again. The combined methanolic extracts were concentrated to 100–150 mL, pooled with other concentrated extracts, and stored at 5°C. Within 2 days, some of the *Alternaria* mycotoxins had precipitated. After filtration, the precipitated fraction was designated as the D fraction. The filtrate was extracted twice with equal volumes of methylene chloride. After concentration, the neutral methylene chloride extract was designated as the B fraction. The aqueous solution was adjusted to pH 2.0 and extracted twice with equal volumes of methylene chloride. The acidic methylene chloride extract was designated as the C fraction and contained primarily TA and some acidic *Alternaria* metabolites (8).

Semipreparative HPLC of Alternaria Mycotoxins

Milligram quantities of *Alternaria* mycotoxins were prepared on a Porasil A column which was equilibrated with 150 mL hexane before use. A Milton Roy pump (Laboratory Data Control, Riviera Beach, FL) was used for solvent delivery. In a typical experiment, 1 g crude extract (B fraction) in 2 mL ethyl acetate was used. To introduce the sample into the column, the solvent line was primed with 2 mL ethyl acetate and the sample was pumped on the column followed by an additional 2 mL ethyl acetate. This step was necessary to avoid any precipitation of the sample which might occur during its introduction. After elution with 4 column volumes of hexane (28 mL per column), stepwise elutions with 620 mL ethyl acetate–hexane (10 + 90), 1210 mL ethyl acetate–hexane (25 + 75), and 500 mL ethyl acetate–hexane (75 + 25) were carried out. The column was subsequently washed with 300 mL ethyl acetate and 500 mL methanol before re-equilibration with 100 mL ethyl acetate–hexane (75 + 25), 100 mL ethyl acetate–hexane (25 + 75), and 150 mL hexane, sequentially, before its next use. Flow rate was maintained at 4.43 mL/min, and 13.3 mL fractions were collected. After thin layer chromatographic (TLC) analysis of the individual fractions, tubes containing pure or like mixtures of mycotoxins were pooled.

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Preparative HPLC of Alternaria Mycotoxins in PrepPak-500 Silica Cartridges

Gram quantities of *Alternaria* mycotoxins were prepared on a PrepLC/System 500 preparative liquid chromatograph (Waters Associates, Milford, MA) using either 1 or 2 PrepPak silica cartridges for each experiment. Before injection of the sample, the column was purged with 2 L hexane, recycled with hexane for 10 min at a flow rate of 500 mL/min, and then quenched with 2 L ethyl acetate–hexane (10 + 90). The column was flushed with 2 L hexane and the hexane was recycled a final 10 min. In a typical experiment, 2 columns arranged in series were used. Approximately 30 g crude extract (B fraction) in 100 mL ethyl acetate was injected onto the column. Stepwise elutions with 4 L hexane, 12 L ethyl acetate–hexane (10 + 90), 14 L ethyl acetate–hexane (25 + 75), 16.5 L ethyl acetate–hexane (50 + 50), and 9 L ethyl acetate were then carried out. Flow rate was 250 mL/min and 100 mL fractions were collected. After TLC analysis, the fractions containing pure or like mixtures of toxins were pooled into 16 large fractions designated by the letters A through P. Some mycotoxins were crystallized after removal of the solvents.

TLC Analysis

TLC analysis was carried out to determine the different *Alternaria* mycotoxins or metabolites present in each fraction after chromatography, and to determine the purity of each preparation. In general, silica gel GF coated to 250 μm thickness or precoated plates were used. Plates were developed in toluene–ethyl acetate–formic acid (5 + 4 + 1) according to Seitz et al. (14). After development, spots of different *Alternaria* mycotoxins and metabolites were visualized under both long and short wavelength UV light.

HPLC Analysis of Alternaria Mycotoxins

Major *Alternaria* mycotoxins obtained from different pooled fractions after chromatography were quantitatively analyzed on a Beckman Model 322 MP gradient liquid chromatograph with a 420 microprocessor, system controller, 2

Table 1. Chromatography of 30 crude *Alternaria* mycotoxins on PrepPak-500 silica cartridge [a]

Fractions (bottle No.)	Major toxins, g/fraction			Minor toxins, mg/fraction			Others [b]
	AME	AOH	ALT	AT-II	AT-I	AS	
A (4–8)							B(0.95)
B (30–54)							G(0.92)
C (55–75)							BG(0.88)
D (76–105)	0.11						
E (106–150)	0.19						
F (151–165)		0.02		1.5	2.0		Y(0.88), YG(0.68)
G (166–195)		0.44		63.7	20.0		
H (196–220)		0.01		13.0	2.0		
I (221–240)				35.0	10.43		
J (241–288)					28.0	26.8	
K (289–308)					23.0	24.7	OY(0.94)
L (309–326)							G(0.44), OY(0.94)
M (327–383)			2.3				
N (384–432)			0.36				
O (433–475)							B(0.36)
P (487–517)							

[a] Fraction B of crude extract which is rich in ALT was used in this experiment.

[b] The abbreviations used in this column indicate the color of fluorescence spot and the values in parentheses are the R_f values of such spots: B = blue, G = green, Y = yellow, OY = orange-yellow.

Model 100A pumps, and a Waters Associates Model 440 dual wavelength absorbance detector. A Shimadzu Chromatopac C-R1A recording data processor (Shimadzu Scientific Instruments, Columbia, MD) was also incorporated into the system. All major *Alternaria* mycotoxins were separated on an Ultrasphere-ODS C_{18} reverse phase column, using the following solvent program: methanol–1.5% acetic acid (50 + 50) for 15 min; then a linear increase in methanol concentration to 65% over 30 min; followed by a linear increase to 100% over 15 min. In addition, the flow rate was programmed: 0.5 mL/min to start, increasing to 1.0 mL/min over 1 min beginning at 14 min, and an increase to 1.5 mL/min over 1 min beginning at 39 min. Information regarding the coefficient of toxin concentration (μg/mL) vs peak areas as monitored at 254 nm as well as retention times for all standard toxins were entered into the data processor so that the concentrations of the unknown samples could be printed out directly after each analysis. Details of this method will be described elsewhere.

Results

Results for the separation of different *Alternaria* metabolites on the semipreparative HPLC column as analyzed by TLC are shown in Figure 1. Three major *Alternaria* mycotoxins, AME, AOH, and ALT, were separated after a single chromatographic step. The solvents in fractions D and E were removed by evaporation. AME was crystallized from these fractions after being dissolved in hot benzene and allowed to stand. After removal of all solvents from fractions H and N, the materials were redissolved in small amounts of ethyl acetate. AOH and ALT, respectively, were crystallized from these fractions after addition of hexane to the ethyl acetate concentrates. Other minor *Alternaria* toxins were distributed in fractions J–M and were eluted from the column in the sequence as AT-II, AT-I, and AS. Because we were interested in the isolation of gram quantities of *Alternaria* metabolites in the PrepLC/System 500, no quantitative HPLC determination of different mycotoxins in each of the pooled fractions was made. However, solvent systems developed for the separation of different *Alternaria* mycotoxins in this experiment served as a guide for the preparative runs.

Three experiments for the preparation of gram quantities of AME, AOH, and ALT with the PrepLC/System 500 were carried out. In the first experiment, 1 cartridge and 15 g sample (crude extract, B fraction) in 30 mL ethyl acetate were used. In the second and third experiments, either 30 g of crude extract B fraction (rich in ALT) or 15 g crude D fraction (rich in AME) in 100 mL ethyl acetate was applied to 2 cartridges connected in series. The solvent systems and programs of these experiments were similar except that less solvent was used in the initial 1-cartridge run. Elution patterns for the 3 experiments were similar and also were qualitatively similar to patterns obtained in the semipreparative experiments, but the resolution of the different toxins in the preparative run was better

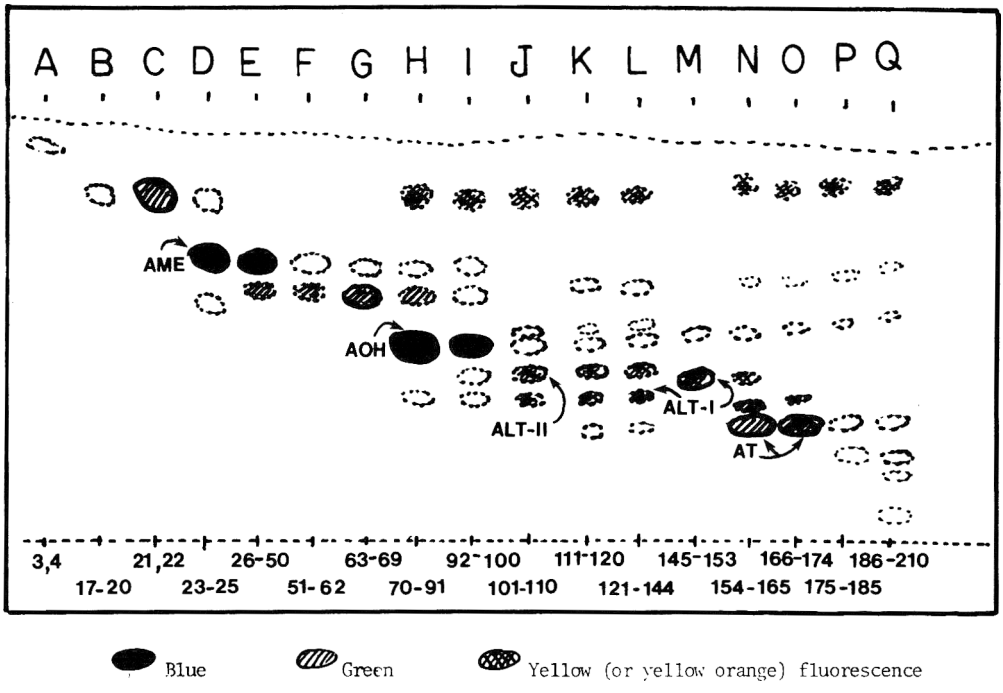

Figure 1. Thin layer chromatography of *Alternaria* metabolites obtained from different fractions after semipreparative HPLC.

A dotted line indicates weak fluorescence spot or minor component. Numbers shown at bottom are tubes pooled. Each tube 1-20 contained 10.0 mL solvent, and each tube 21-210 contained 13.3 mL solvent. The column was equilibrated with hexane, and was eluted with hexane, tubes 1-8; ethyl acetate-hexane (10 + 90), tubes 9-58; (25 + 75), tubes 59-510; (75 + 25), 151-187; and ethyl acetate, tubes 188-210.

than that for the semipreparative run. AME and AOH crystallized in some of the fractions after standing briefly at room temperature. Each of the pooled fractions was quantitatively analyzed for different *Alternaria* toxins by analytical HPLC, and the results are given in Table 1. The 3 major toxins were well separated from each other as well as from other contaminants. After evaporation of the solvents from fractions E, G, and M, different mycotoxins were crystallized in the solvent systems described in the semipreparative runs. A total of 0.2, 0.4, and 1.8 g crystalline AME, AOH, and ALT, respectively, were obtained from those fractions. TLC and HPLC analyses indicated that the purity of these preparations was greater than 95%. Mass spectral analyses revealed that the purified AME, AOH, and ALT all have molecular ion peaks corresponding to the reported values.

While complete resolution of the minor *Alternaria* toxins was not achieved even in the preparative runs, some separation of those toxins was observed. For example, fraction H contained primarily AT-II, fraction I contained both AT-II (major) and AT-I, and fraction J contained AT-I and AS. However, large quantities of AT-II and AT-I were associated with fraction G which primarily contained AOH.

Discussion

Results obtained from the present study demonstrate that preparative HPLC is an effective method for isolating large quantities of pure *Alternaria* mycotoxins for toxicity studies. The entire chromatographic separation requires less than 1 working day, thus saving considerable time and effort. The solvents used in the study are not only economical, but relatively safe as well. This contrasts with 2 methods reported earlier, which in 1 case involved the lengthy classic solvent partition and repeated crystallization (8, 9), and in the other case involved a chromatographic procedure using benzene and tetrahydrofuran (THF) as the elution solvents (5, 10).

Although the minor *Alternaria* mycotoxins (AT-I, AT-II, and AS) cannot be isolated in pure form by the present method because of the

minute amount of these toxins present, this method at least can serve as a first step in the isolation of these compounds. TLC analysis also revealed that some fractions contained only 1 or 2 fluorescent substances (Figure 1) which require further study for identification and characterization. An additional advantage of the present method is its use for the isolation of some as yet unidentified compounds.

The investment in instrumentation is perhaps the major limiting factor for the present method. Nevertheless, the solvent systems developed in this study could be readily applied to any other chromatographic system using silica gel as adsorbent. In both the semipreparative and preparative runs, AME eluted from the column with ethyl acetate–hexane (10 + 90), while AOH and the other minor metabolites eluted with ethyl acetate–hexane (25 + 75). This is comparable to the conditions developed by Pero and Main (5) in which they used benzene and THF as the eluting solvents in silica gel chromatography. These authors demonstrated that AME and AOH were eluted from the column with 5% and 15% THF in benzene, respectively. Although ALT eluted from the semipreparative column with ethyl acetate–hexane (75 + 25), subsequent preparative studies showed that ALT could be recovered with ethyl acetate–hexane (50 + 50). TLC analysis of the fractions obtained by both methods indicated that the fractions obtained from the preparative column had fewer contaminants. Therefore, we recommend that ethyl acetate–hexane (50 + 50) be used for isolating ALT by semipreparative chromatography or by any other alternative chromatography. To prepare pure ALT, Pero et al. (10) used 50% THF in benzene to elute ALT from the silica gel column.

Preparation of gram quantities of mycotoxins by HPLC has been demonstrated for ochratoxin A (15), and a single solvent system was used in such a study. In view of the complexity of metabolites elaborated by fungi and the fact that often several major metabolites are produced, the present approach of stepwise elution with solvent mixtures of increasing polarity should be useful in isolating potentially hazardous toxic metabolites which are difficult to obtain by other methods.

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Potential Mold Growth, Aflatoxin Production, and Antimycotic Activity of Selected Natural Spices and Herbs

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Ground spices and herbs are evaluated as substrates for mycelial growth, sporulation, and aflatoxin production. Three toxigenic strains of Aspergilli, *A. flavus* ATCC 15548, *A. flavus* NRRL 3251, and *A. parasiticus* NRRL 2999, were cultured on moist, commercially packaged herbs and spices. All substrates used were ground and included thyme, celery seed, oregano, cinnamon, ginger, caraway seed, clove, mustard, sesame seed, and rosemary leaves. Following inoculation of the natural materials in sterile bottles containing sterile water, the cultures were incubated 30 days at 23 ± 4°C. Not all strains of Aspergilli grew, sporulated, or produced toxins. There were definite strain differences and definite substrate differences for the variables evaluated. Sesame seed produced toxins B_1, G_1, and G_2, with a mean of 167 ppm for 3 strains. *A. flavus* ATCC 15548 was the greatest overall toxin producer followed by *A. parasiticus* NRRL 2999 and *A. flavus* NRRL 3251. Ginger and rosemary leaves were also substantial producer-substrates. Mustard, caraway seed, and celery seed were judged as intermediate-producing substrates. Absolute antimycotic substrates were cinnamon and clove. Antiaflatoxigenic substrates were thyme and oregano. Mustard also may be antimycotic. Aflatoxins B_1 and G_1 were the more commonly found toxins.

Aflatoxins are fungal metabolites contaminating many food products, especially those that are often stored, such as peanuts, grains, and cereals. The natural occurrence of aflatoxins in herbs and spices has become of increasing interest because of the widespread use of these substances in the world today (1).

Cinnamon reportedly prohibits toxin production (2–4). Hitokoto et al. (5, 6) demonstrated that cinnamon as well as pepper inhibit aflatoxin production. It has been established that ground cinnamon contains a natural antimycotic substance (3, 7–11). The presence of aflatoxins in black pepper, celery seed, and nutmeg has been reported by Suzuki et al. (12). Scott and Kennedy (13, 14) found aflatoxins in coriander, ginger, nutmeg, and turmeric. There are 2 factors which may contribute to toxin production: many spices are grown and processed in warm tropical climates which are favorable for fungal growth (15), and harvest and drying procedures may allow for high moisture content.

For the purpose of this study, some of the spices and herbs already evaluated for mycotoxin production or antimycotic activity, and some that have not been examined, have been included. Those spice or herb substrates reported here include celery seed, cinnamon, caraway seed, ginger, mustard, rosemary leaves, thyme, oregano, sesame seed, and clove. Analytical procedures used were compared for applicability to individual foodstuffs tested.

Experimental

Spices and Herbs

Ground, commercially packaged spices and herbs from 4 sources were used: Thyme, oregano, cinnamon, ginger, and clove were products of C. F. Sauer Co., Richmond, VA; sesame seed and rosemary leaves were packaged by McCormick and Co., Inc., Baltimore, MD; celery seed and caraway seed were from Spice Island—Specialty Brands, Inc., San Francisco, CA.

Culture Preparation

Aseptic techniques were used to remove 1.5 g aliquots from the commercial packages. Spices that were not commercially ground such as celery seed and rosemary leaves were ground with a glass mortar and pestle. Sesame seed and caraway seed were ground, but did not lend themselves to as fine a consistency.

Triplicate samples and controls were made for each substrate and each mold tested. Glass prescription bottles (2 oz) with 1 flat side and 1 rounded side were filled with various volumes of triple-distilled water, plugged with cotton, and autoclaved 20 min at 121°C, 15 psi. After cooling to room temperature, these sterile bottles, water, and plugs received specific weights of each spice or herb. Water and substrate were mixed to form a wet slurry which would flow very slowly. The herbs and spices were transferred under a hood previously cleaned with 25% bleach and exposed to ultraviolet light for 30 min. The slurry was allowed to sit 30 min to

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Table 1. Mean percent of culture surface area covered with mycelium [a]

Spice or herb	Strain [b]	Days following inoculation 2	7	14	30
Thyme	3251	—	22	30	33
	2999	51	77	77	80
	15548	10	50	90	100
Celery seed	3251	—	—	30	53
	2999	—	—	—	77
	15548	—	—	—	58
Caraway seed	3251	—	100	100	100
	2999	93	100	100	100
	15548	17	100	100	100
Oregano	3251	—	—	—	37
	2999	—	—	—	25
	15548	—	—	—	—
Rosemary leaves	3251	35	100	100	100
	2999	20	95	95	100
	15548	3	100	100	100
Sesame seed	3251	—	100	100	100
	2999	—	33	33	33
	15548	23	100	100	100
Ginger	3251	50	90	100	100
	2999	98	100	100	100
	15548	—	100	100	100
Mustard	3251	—	—	—	—
	2999	—	—	—	—
	15548	—	—	—	10

[a] No detectable growth shown by any strain on cinnamon or clove during 30 day study.
[b] *A. flavus* NRRL 3251, *A. parasiticus* NRRL 2999, *A. flavus* ATCC 15548.

allow further water uptake by the herb or spice. Then the spices and herbs were inoculated with specific aflatoxigenic strains of mold. The weight of each substrate (1.5 g) added was predetermined in preliminary moisture and substrate ratio studies to give the desired slurry. Cinnamon, mustard, oregano, and thyme were each placed in bottles containing 5.5 mL triple-distilled, sterile water. Ginger and clove were placed in 4.5 mL sterile distilled water. Caraway seed, celery seed, and sesame seed were placed in 4 mL sterile, triple-distilled water, and rosemary leaves were placed in the bottle containing 8 mL sterile distilled water.

Inoculations and Incubation

Three aflatoxigenic strains were used: *A. flavus* ATCC 15548, *A. flavus* NRRL 3251, and *A. parasiticus* NRRL 2999. The strains were obtained from stock cultures maintained in our laboratory on potato-dextrose agar slants. Each spice or herb was inoculated with 1 microloopful of spores from each strain. After inoculation, all samples were stored on trays in a dark cabinet at room temperature (23 ± 4°C) for 30 days. These samples were observed regularly for mold growth and spore formation.

Surface Area Measurements

Culture vials were viewed from above with a transparent grid having 100 squares placed over the area of the bottle or substrate surface. This was used to estimate the area of mycelial growth and subsequent area covered by spores. The first reading was made after 54 h with regular daily readings thereafter for 14 days, and then at several day intervals until the 30th day.

Aflatoxin Analysis

Individual culture bottles containing substrate, mycelia, and spores were extracted and analyzed

Table 2. Mean percent of mycelial area covered with spores [a]

Spice or herb	Strain [b]	Days following inoculation 5	6	7	10	11	12	15	30
Thyme	3251	—	—	—	—	—	—	—	—
	2999	—	—	100	100	100	100	100	100
	15548	—	—	—	—	52	100	100	100
Caraway seed	3251	—	100	100	100	100	100	100	100
	2999	100	100	100	100	100	100	100	100
	1548	—	100	100	100	100	100	100	100
Rosemary leaves	3251	100	100	100	100	100	100	100	100
	2999	67	100	100	100	100	100	100	100
	15548	—	100	100	100	100	100	100	100
Sesame seed	3251	—	17	33	100	100	100	100	100
	2999	33	80	100	100	100	100	100	100
	15548	17	32	67	82	92	92	92	100
Ginger	3251	—	—	—	—	100	100	100	100
	2999	100	100	100	100	100	100	100	100
	15548	—	82	100	100	100	100	100	100

[a] No spore formation detected for celery seed, oregano, cinnamon, clove, or mustard during 30 day study.
[b] *A. flavus* NRRL 3251, *A. parasiticus* NRRL 2999, *A. flavus* ATCC 15548.

Table 3. Mean aflatoxin levels (ppb) with standard deviations for 3 Aspergilli isolates grown on moist spice and herb substrates [a,b]

Toxin	*A. parasiticus* NRRL 2999	*A. flavus* NRRL 3251	*A. flavus* ATCC 15548	Mean ± SD (overall total)
		Ground Celery Seed[c]		
Aflatoxin B_1	280.6 ± 108.8	ND[d]	197.2 ± 153.7	159.3 ± 144.1
		Ground Rosemary Leaves[e]		
Aflatoxin B_1	164.8 ± 112.2	133.3 ± 115.5	2685.2 ± 0.0	994.4 ± 1464.3
		Ground Ginger[f]		
Aflatoxin B_1	ND	ND	14 867.0 ± 1414.2	
Aflatoxin G_1	ND	ND	22 847.0 ± 2258.5	12 629.7 ± 21 875.2
Aflatoxin G_2	ND	ND	175.0 ± 0.0	
Total	ND	ND	37 889.0	
		Ground Sesame Seed[g]		
Aflatoxin B_1	6311.7 ± 3501.5	ND	136 112.9 ± 92 258.2	
Aflatoxin G_1	13 334.0 ± 5774.1	ND	341 724.5 ± 256 557.5	166 938.8 ± 272 310.0
Aflatoxin G_2	ND	ND	3333.3 ± 1443.4	
Total	19 645.7	ND	481 170.7	
		Ground Mustard[h]		
Aflatoxin B_1	ND	ND	583.9 ± 0.0	
Aflatoxin G_1	ND	ND	583.9 ± 0.0	389.6 ± 673.9
Total	ND	ND	1167.8	
		Ground Caraway Seed[i]		
Aflatoxin B_1	88.9 ± 0.0	ND	150.0 ± 0.0	
Aflatoxin G_1	55.6 ± 0.0	ND	241.7 ± 43.2	178.7 ± 198.1
Total	144.5	ND	391.7	

[a] Triplicate cultures were tested 3 times each. Values are based on original "dry" weight of spice substrate used.
[b] Ground cloves (1.5 g with 4.5 mL water), ground cinnamon (1.5 g with 4.0 mL water), ground oregano (1.5 g with 5.5 mL water), and ground thyme (1.5 g with 5.5 mL water) were antiaflatoxigenic.
[c] 1.5 g celery seed with 4.0 mL water.
[d] None detected. Levels as low as 2 ppb may be detected using this system.
[e] 1.5 g rosemary leaves with 8.0 mL water.
[f] 1.5 g ground ginger with 4.0 mL water.
[g] 1.5 g ground sesame seed with 4.0 mL water.
[h] 1.5 g ground mustard with 5.5 mL water.
[i] 1.5 g ground caraway seed with 4.0 mL water.

together. Methylene chloride (100 mL) and water (5 mL) were added in proportion to the original dry weight added as substrate. All steps followed AOAC methods (16). Each bottle was capped and shaken vigorously on a mechanical shaker for 1 h. Following gravity filtrations through paper, an aliquot of the filtrate was applied to the resin column, eluted, evaporated, and diluted to volume in benzene containing 2% acetonitrile. Triplicate aliquots of each extracted culture were subjected to thin layer chromatography (TLC) on Adsorbosil-1 silica gel plates again according to AOAC methods (16). The data are reported as ppb dry weight of the bottle contents. Separate dry samples of each spice or herb substrate used in the study were tested to confirm the absence of aflatoxins before experimental use. The analyses were completed at the Mycotoxin Laboratory, Virginia Division of Consolidated Laboratories, Richmond, VA.

Data Analysis

All data were accumulated via triplicate samples and triplicate readings. Means and standard deviations were developed. Duncan's New Multiple Range Test was applied to the data ($P = 0.05$).

Results

Mycelial Growth and Sporulation

A. flavus NRRL 3251 showed mycelial growth within 48 h on rosemary leaves and ginger. By

Table 4. Aflatoxigenic rankings for the spices and herbs and the 3 Aspergilli isolates used [a]

Rank	*A. parasiticus* NRRL 2999	*A. flavus* NRRL 3251	*A. flavus* ATCC 15548	Overall
1	sesame seed	rosemary leaves	sesame seed	sesame seed
2	celery seed	—	ginger	ginger
3	rosemary leaves	—	rosemary leaves	rosemary leaves
4	caraway seed	—	mustard	mustard
5	—	—	caraway seed	caraway seed
6	—	—	celery seed	celery seed

[a] Substrates not listed had no detectable aflatoxins and include: oregano, thyme, cloves, and cinnamon.

the end of the first week all cultures exhibited growth except oregano, cinnamon, clove, and mustard. After 30 days, all cultures except thyme, celery seed, and oregano, which had produced growth, had 100% sporulation. Cinnamon, clove, and mustard failed to support any detectable growth (Tables 1 and 2).

A. parasiticus NRRL 2999 showed growth within the first 48 h on thyme, caraway seed, rosemary leaves, and ginger. By the end of the first week, caraway seed, rosemary leaves, and ginger had 95% of area covered with growth; thyme, caraway seed, rosemary leaves, sesame seed, and ginger had reached 100% sporulation. By the final reading, all cultures showed growth except cinnamon, clove, and mustard.

A. flavus ATCC 15548 showed growth on thyme, caraway seed, rosemary leaves, and sesame seed within the first 48 h. By the end of the first week caraway seed, rosemary leaves, and ginger all had complete surface area growth and sporulation. Sesame seed attained 100% mycelial coverage with 67% spore formation. Oregano, celery seed, cinnamon, mustard, and clove showed no detectable growth. By the final reading, oregano, cinnamon, and clove were still free of detectable growth, but sesame seed had 100% mycelial coverage and complete sporulation coverage. Mustard exhibited slight mycelial growth.

Aflatoxin Production

A. flavus ATCC 15548 produced the greatest quantity of toxin and was also a producer on 6 of the 10 substrates. *A. parasiticus* NRRL 2999 produced toxin on 4 of the 10 substrates but at lower levels than the above strain. For the substrates tested, *A. flavus* NRRL 3251 produced toxin in the lowest level and only on one substrate, rosemary leaves (Tables 3 and 4). Sesame seed was by far the best substrate providing high levels of toxin, and rosemary leaves was the only substrate conducive to toxin production by all 3 strains tested. The following substrates were antiaflatoxigenic: cinnamon, clove, oregano, and thyme. Mustard and ginger only supported toxin production when strain 15548 was used.

Cinnamon, clove, and possibly mustard may be classified as antimycogenic substrates; they supported essentially no mycelial growth (Table 5). Oregano and celery seed supported growth poorly, which was evident only after 2 weeks. Oregano and celery seed cultures failed to sporulate.

Discussion

It is evident that certain spices, especially cinnamon, cloves, and possibly oregano and mustard inhibit mycelial growth and subsequent toxin production for the isolates tested in this study. Other investigators have reported the inhibitory nature of cinnamon (2–6). Very recently Hitokoto et al. reported that clove, anise, and allspice completely inhibited fungal growth of *A. flavus* and *A. versicolor* (17). Our study confirmed both the antimycogenic and antitoxigenic influence of cinnamon and clove, and added thyme, oregano, and possibly mustard to this group. We also found some spices or herbs that were highly aflatoxigenic.

Major factors that may influence mycelial growth and toxin production are absence of necessary nutrients from the substrate, competing microorganisms in the substrate, inhibitory chemicals, and of course the natural barriers such as seed coat, water content, pH, temperature, and length of time for the mold to grow.

Morozumi (18) has identified the chemical inhibitor in cinnamon as *o*-methyoxycinnamaldehyde. Hitokoto et al. (17) recently reported that eugenol from cloves and thymol from thyme were inhibitory agents. These reports were based on the introduction of specific amounts of spice extracts into standard mold culture media for growth periods up to 12 days. Our study used the moist natural ground spice or herb as the sole substrate for the mold growth for 30 days at room temperature.

We assume that there were sufficient carbon compounds present in all the substrates to support fungal growth (19). The levels of metals in

Table 5. Antimycotic, antisporogenic, antiaflatoxigenic index based on rankings of spices and herbs used as substrate for 3 Aspergilli isolates

	A. parasiticus NRRL 2999			*A. flavus* NRRL 3251			*A. flavus* ATCC 15548			Overall total for all isolates			
Rank	Mycelial growth[a]	Sporulation[b]	Total toxin[c]	Mycelial growth	Sporulation	Total toxin	Mycelial growth	Sporulation	Total toxin	Mycelial growth	Sporulation	Total toxin	Descriptive summary
1	cinnamon[d]	cinnamon	cinnamon	cinnamon	cinnamon	caraway seed	cinnamon	celery seed	cinnamon	cinnamon	celery seed	cloves	antimycogenic
2	cloves	celery seed	cloves	cloves	celery seed	celery seed	cloves	cinnamon	cloves	cloves	cinnamon	cinnamon	antisporogenic
3	mustard	cloves	ginger	mustard	cloves	cinnamon	mustard	cloves	oregano	mustard	cloves	oregano	antiaflatoxigenic
4	oregano	mustard	mustard	thyme	mustard	cloves	oregano	mustard	thyme	oregano	mustard	thyme	
5	sesame seed	oregano	oregano	oregano	oregano	ginger	celery seed	oregano	celery seed	thyme	oregano	celery seed	
6	celery seed	caraway seed	thyme	celery seed	thyme	mustard	caraway seed	caraway seed	caraway seed	celery seed	thyme	caraway seed	
7	thyme	ginger	caraway seed	caraway seed	caraway seed	oregano	ginger	ginger	mustard	sesame seed	caraway seed	mustard	
8	caraway seed	rosemary leaves	rosemary leaves	ginger	ginger	sesame seed	rosemary leaves	rosemary leaves	rosemary leaves	caraway seed	ginger	rosemary leaves	mycogenic
9	ginger	sesame seed	celery seed	rosemary leaves	rosemary leaves	thyme	sesame seed	sesame seed	ginger	ginger	rosemary leaves	ginger	sporogenic
10	rosemary leaves	thyme	sesame seed	sesame seed	sesame seed	rosemary leaves	thyme	thyme	sesame seed	rosemary leaves	sesame seed	sesame seed	aflatoxigenic

[a] Mycelial growth as % area covered after 30 days.
[b] % of mycelium covered with spores after 30 days.
[c] Total aflatoxins found in substrates after 30 days and based on original dry weight of substrate.
[d] Left vertical line shows substrates having equal ranking.

the substrates may have been important because studies have reported the need for zinc for fungal growth (20).

It is our contention that the active chemical(s) in the spice or herb was the contributing factor as an inhibitory agent. Further identification of these chemicals and the confirmation of their actual activity will be needed and are planned. It is also evident that spices and herbs are not all antimycotic or even antiaflatoxigenic. For example, sesame seed, ginger, and rosemary leaves could readily be classified as very supportive for growth, sporulation, and toxin production. Under these laboratory conditions, the total aflatoxins produced on these substrates by some or all of the Aspergilli tested were in the ppm range, which is high for foods in general (21, 22).

The spices and herbs merit further study based on their antimycotic activity, their antitoxigenic potential, and the potential that they could be contaminated naturally by mycotoxins. There was a tendency in our study for the established and consistent aflatoxigenic strains such as 2999 and 15548 (21, 22) not to produce all 4 toxins, B_1, B_2, G_1, G_2, but to favor B_1 and G_1. This tends to support the antitoxigenic character of the substrate. There is also evidence of different antimycotic and antitoxigenic responses to the fungal isolates. Only 15548 produced toxin on ginger and mustard. Mycelia on the ground mustard were not highly evident on the surface, but repeat studies, growing for a longer time period, indicated that both mustard and ginger allowed only a selected strain to produce toxin. Since mustard never sporulated, even in repeat studies, we believe it has antimycotic activity. Aflatoxins B_1, G_1, and G_2 were all produced by 15548 on ginger, and in such high levels that ginger appears to be conducive to aflatoxin occurrence. The FDA laboratories and others apparently are aware of this potential and are developing better extraction and cleanup procedures for ginger analysis.

Generally, the AOAC analytical procedures that were followed seemed adequate for the spices and herbs tested for aflatoxins (16). Specific and improved procedures could be developed for each substrate. Such methodology should provide for better determinations if needed.

In summary, we recommend that sesame seed, ginger, and rosemary leaves be monitored for natural aflatoxin concentration and that oregano, clove, thyme, and cinnamon be given little or no attention for mycotoxin occurrence but be studied for their application as antimycotic and antitoxigenic agents.

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Rapid, Economical Method for Determination of Aflatoxin and Ochratoxin in Animal Feedstuffs

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A quantitative procedure widely used in European Economic Community (EEC) countries has been successfully scaled down to produce a rapid method for determination of aflatoxin B_1 (and other aflatoxins) in animal feeds. Without modification, the method may be used for simultaneous ochratoxin A determination in simple feeds, but a slightly different extraction procedure is required for compound feeds. Validity of the method has been demonstrated by comparison with the full EEC procedure for aflatoxin B_1 and the Nesheim method for ochratoxin A. Analyses may be completed within 2 h and there is a considerable savings in materials over the 2 reference methods. The procedure is also less hazardous because volumes of toxic extract are small, and the operator is exposed to minimum solvent vapor.

Speed and economy are 2 increasingly important factors in the analysis of animal feedstuffs for mycotoxins. Most current methods are time-consuming, mainly in the extraction and cleanup steps, and costly because they use large volumes of expensive solvents such as chloroform.

The present report describes a quantitative procedure for determining aflatoxin B_1, which is considerably faster and cheaper than a method widely used in the European Economic Community (EEC) (1). Also, the method can be used to determine aflatoxins B_2, G_1, and G_2, and can be adapted for ochratoxin A. This development arose from our unpublished observations that the original method (1) could be modified for ochratoxin A and that the silica gel chromatography step could be scaled down satisfactorily using Sep-Pak silica cartridges, thus saving operator time and expensive solvents.

Determinations of aflatoxin B_1 and ochratoxin A in samples of several different naturally contaminated animal feedstuffs have been compared with results obtained on the same samples by the original aflatoxin method (1) and the Nesheim method (2) for ochratoxin A.

METHOD

Extraction

Weigh 10 g finely ground sample of feedstuff into wide-mouth 6 oz bottle with 5 g diatomaceous earth. Add 5 mL water and 50 mL $CHCl_3$ and seal bottle with aluminum-lined screw cap. Extract toxins 30 min in wrist-action shaker. Filter through Whatman No. 41 paper, dry extract by adding 2 g anhydrous Na_2SO_4 with gentle mixing 2 min, and refilter.

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Cleanup

Mix 5 mL aliquot with 5 mL *n*-hexane and, using 50 mL hypodermic syringe with Luer fittings, pass through Sep-Pak silica cartridge (Waters Associates, Inc., Milford, MA). Wash silica with 5 mL dry ethyl ether and elute aflatoxin with 20 mL $CHCl_3$–methanol (19 + 1). Transfer to 50 mL round-bottom flask and evaporate to dryness in rotary evaporator in stream of nitrogen. Redissolve in $CHCl_3$ and transfer quantitatively to 2 mL glass vial. Evaporate solvent under nitrogen and redissolve residue in 100 μL $CHCl_3$.

Thin Layer Chromatography

Aflatoxin B_1.—For quantitative 2-dimensional TLC, use 10 × 10 cm aluminum sheets coated with silica gel 60 (Merck, art. 5553). Develop chromatogram in lined, equilibrated tanks with ethyl ether–methanol–water (94 + 4.5 + 1.5) (direction 1) and with $CHCl_3$–acetone (90 + 10) (direction 2). Quantitate aflatoxin B_1 by comparing, visually or densitometrically under UV light at 360 nm, standard spots of 0.5, 1, 2, and 3 ng aflatoxin B_1 with an appropriate aliquot (usually 5 μL) of the extract, diluted if necessary. Confirm by co-chromatography with added standard, sulfuric acid spray, and conversion to hemiacetal (1).

The 4 aflatoxins B_1, B_2, G_1, and G_2 are resolved by this solvent pair and may be quantitated on separate chromatograms, using appropriate dilutions of extract.

Modification for ochratoxin A.—For simple feeds, use 20 mL methanol–90% formic acid (19 + 1) to elute ochratoxin A from the cartridge after aflatoxins elution.

For compound feeds, use a mixture of 5 mL 0.1M phosphoric acid and 50 mL $CHCl_3$ (2) in place of water–$CHCl_3$ extractant. Develop 2-dimensional chromatograms in lined, equilibrated tanks with toluene–ethyl acetate–90% formic acid (60 + 30 + 10) (direction 1) and

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Table 1. Aflatoxin B_1 determinations in animal feeds by 2 methods (μg/kg)

Animal feed	EEC method (1) Obs. 1	EEC method (1) Obs. 2	Present method Obs. 1	Present method Obs. 2	Mean value as % of EEC method (1)[a]
Simple:					
1. Groundnut meal	5000	5000	5000	4375	
	4375	4375	4375	4375	96.7
2. Cotton seed meal	200	200	175	175	
	200	200	200	200	94
3. Copra	<10	<10	<10	<10	
	2.5	2.5	3.5	3.5	140
Compound:					
4. Calf feed	25	25	20	20	
	22.5	22.5	20	20	83.3
5. Dairy ration A	35	35	30	30	
	35	35	35	35	94.3
6. Dairy ration B	800	800	875	1000	
	700	700	750	875	116.7

[a] Detection limit 1 μg/kg in simple and compound feeds: recoveries of added aflatoxin B_1 (50 μg/kg) were ca 95%.

$CHCl_3$–acetone (90 + 10) (direction 2). To ensure baseline resolution in direction 2, carry out second development before aqueous component of solvent 1 dries completely, thus conferring acidity on solvent 2. This is achieved by allowing only 10 min (in fume hood) for evaporation of solvent 1.

For quantitation, compare standards of 5, 10, 20, and 30 ng ochratoxin A. Confirm by conversion to ochratoxin A by acid hydrolysis, to its acetate (3), or to the ethyl ester (4).

Results and Discussion

Aflatoxin B_1 concentrations estimated by this and by the standard EEC method (1) from which it is derived are given in Table 1. Analyses were carried out in duplicate on 3 simple and 3 compound feeds, all naturally contaminated. TLC results were quantitated by visual comparison with standards, and the estimates of 2 observers are included. The proposed modification appears to be as reproducible as the original procedure; results obtained by the 2 methods agree within experimental error. Indeed, differences on TLC related merely to comparisons with the next lower or higher aflatoxin B_1 standards.

As shown in Table 2, ochratoxin A determinations were also satisfactory for 4 samples of simple feeds; mean values were consistently higher than those obtained by the Nesheim method (2). Recovery of ochratoxin A from the 2 samples of compound feed was evidently incomplete when the chloroform–water extractant was used, but this was improved when water was replaced by 0.1M phosphoric acid (Table 3). Use of the acidic solvent mixture did not adversely

Table 2. Ochratoxin A determinations in animal feeds by 2 methods (μg/kg)

Animal feed	Nesheim method (2) Obs. 1	Nesheim method (2) Obs. 2	Present method Obs. 1	Present method Obs. 2	Mean value as % of Nesheim method (2)
Simple:					
2. Cotton seed meal	75	75	—	—	
	75	75	100	75	117.3
7. Wheat and barley mix	75	75	75	75	
	75	63	100	100	122.2
8. Barley A	1000	1000	750	875	
	750	750	875	1000	100
9. Barley B	1500	1750	2000	2000	
	1250	1250	1500	1500	121.7
Compound:					
4. Calf feed	750	750	175	175	
	750	750	175	175	23.3
10. Cattle feed	100	88	38	38	
	150	150	38	25	28.7

Table 3. Aflatoxin B_1 and ochratoxin A determinations in compound feeds by the present method using acidic extractant

Mycotoxin	Animal feed	μg Mycotoxin/kg Obs. 1	μg Mycotoxin/kg Obs. 2	Mean value as % of reference method[a]
Aflatoxin B_1[b]	4. Calf feed	20	30	
		20	20	94.7
	6. Dairy ration B	750	750	
		875	938	110.4
Ochratoxin A[c]	4. Calf feed	750	750	
		880	750	104.3
	10. Cattle feed	75	75	
		88	75	64.1

[a] EEC method (1) for aflatoxin B_1 and the Nesheim method (2) for ochratoxin A.

[b] Detection limit and recovery, see footnote to Table 1.

[c] Detection limit 20 μg/kg in simple and compound feeds. When added at 500 μg/kg, recoveries of ochratoxin A were ca 95% but in compound feeds recoveries are probably poorer at concentrations close to the detection limit. (Compare results for samples 2 and 7 in Table 2 and sample 10 above.)

affect the simultaneous determination of aflatoxin B_1 but this application is not recommended because of potential hemiacetal formation.

The advantages of the method described include: with appropriate extractants, a single procedure may be used to quantitate aflatoxins and ochratoxin A in simple and compound feeds; the method is rapid, taking about 2 h to complete an analysis; the method is economical, with a considerable savings in materials, even allowing for the cost of disposable Sep-Pak cartridges; and the method is less hazardous because it uses smaller volumes of solvent and exposes the operator to less vapor than either of the 2 reference methods. Although the above description includes quantitation by a visual TLC procedure, this detection system can be replaced by fluorodensitometry or high pressure liquid chromatography.

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Determination of Aflatoxins in Animal Tissues

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A method for the determination of aflatoxins in animal tissues has been developed, and applied successfully to beef, swine, chicken, and human livers, and to beef kidney, heart, spleen, muscle, and blood. Blended tissue is denatured with citric acid and extracted with dichloromethane on a wrist-action shaker. After filtration, the extract is partially purified on a silica gel column, and aflatoxins B_1 and M_1 are determined by 2-dimensional thin layer chromatography and densitometry. Recoveries of B_1 and M_1 added to meat tissues and blood were approximately 90 and 80%, respectively. The method gave results for a contaminated freeze-dried liver comparable to analyses by 3 other published meat tissue methods. The method is rapid and has a determination limit ≤0.1 ng/g. In addition, the method uses less toxic and smaller quantities of solvents and chemicals.

Aflatoxins have been found in organs and tissues of beef, swine, and poultry that have ingested aflatoxin-contaminated feeds (1–3). The liver is the target organ of aflatoxicosis; however, B_1, B_2, and M_1 have been detected in other edible animal tissues and, therefore, present a potential health hazard for humans. Aflatoxin levels reported in meats are usually less than 1 ng/g, but animal tissues contaminated with aflatoxin at any detectable level should be diverted from commercial food channels. This requires accurate and sensitive quantitative methods.

Several methods for determining aflatoxins in liver have been published (4–9). These methods either require considerable time, lack the desired detection limit (≤0.1 ng/g), or give final extracts for thin layer chromatography (TLC) that contain fluorescent impurities which make identification of low levels of aflatoxin difficult. A method for the determination of aflatoxin M_1 in dairy products (10) was modified for animal tissues. The method is rapid and sensitive, and gives TLC extracts that are free of interfering contaminants. In addition, the method uses less toxic and smaller quantities of solvents and chemicals. This paper reports the application of this method to beef, swine, chicken, and human livers and to beef kidney, heart, spleen, muscle, and blood.

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Experimental

Apparatus

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

(b) *Meat grinder.*—Waring Blendor, Model EP-1, and any manual food grinder.

(c) *Chromatographic columns.*—Glass 50 × 1.0 cm id; see **26.A10(b)** (11).

(d) *Filter paper.*—32 cm, S&S No. 588, or equivalent rapid flow, high wet-strength paper; and 24 cm, S&S No. 560, or Whatman 2V or equivalent medium flow paper.

(e) *Thin layer plates.*—10 × 10 cm commercial pre-poured, 0.25 mm thickness, glass plates (handcut from 20 × 20 cm) (E. Merck silica gel 60, No. 5763) or prepare in laboratory as described in **26.A10(d)** (11).

Reagents

(a) *Solvents.*—Reagent grade, distilled in glass. Glacial acetic acid, acetone, acetonitrile, benzene, chloroform (0.75% ethanol), dichloromethane, ethyl ether (0.01% ethanol, peroxide-free), hexane (68–69°C), isopropanol, and toluene.

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

(c) *Silica gel for column chromatography.*—E. Merck silica gel 60 (No. 7734) 0.063–0.200 mm (70–230 mesh), or equivalent. Treat as described in **26.A11(c)** (11).

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

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

(f) *Aflatoxin standards.*—Prepare aflatoxins B_1 and M_1 in acetonitrile–benzene (1 + 9) to contain 0.25 μg/mL each for either visual or densitometric analysis. If aflatoxins G_1, B_2, and/or G_2 are needed, prepare G_1 at 0.25 μg/mL and B_2 and G_2 at 0.05 μg/mL. Store standards in 1 dram vials fitted with Teflon-lined screw caps and store at 0°F when not in use.

Samples

Artificially contaminated animal tissues.—All animal tissues and blood samples used in this

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Table 1. Recovery of aflatoxins B_1 and M_1 from artificially contaminated meat products [a]

Meat product	B_1 and M_1 added, ng/g or ng/mL	Aflatoxin B_1				Aflatoxin M_1			
		Recd, ng/g or ng/mL	Rec., %	Std dev.	Coeff. of var., %	Recd, ng/g or ng/mL	Rec., %	Std dev.	Coeff. of var., %
Liver, beef	0.1	0.091	90.7	0.013	14.6	0.084	84.4	0.013	15.4
	0.5	0.476	95.2	0.031	6.6	0.409	81.1	0.036	8.9
	1.0	0.922	92.2	0.132	14.3	0.817	81.7	0.077	9.4
Liver, swine	0.5	0.480	96.0	0.061	12.7	0.420	84.0	0.037	8.8
Liver, chicken	0.5	0.444	88.8	0.029	6.6	0.417	83.3	0.039	9.4
Liver, human	0.5	0.524	104.0	0.050	9.5	0.486	97.3	0.030	6.1
Kidney, beef	0.5	0.476	95.1	0.045	9.6	0.431	86.3	0.069	15.9
Heart, beef	0.5	0.459	91.8	0.049	10.7	0.428	85.6	0.045	10.5
Spleen, beef	0.5	0.474	94.8	0.033	7.0	0.414	82.7	0.027	6.6
Muscle (chuck), beef	0.5	0.443	88.6	0.077	17.4	0.438	87.6	0.048	11.0
Blood (coag.), beef	0.5	0.475	94.9	0.045	9.5	0.464	92.8	0.046	9.9
Blood (uncoag.), beef	0.5	0.418	83.6	0.054	13.0	0.391	78.2	0.052	13.3

[a] As determined by 2-dimensional TLC and densitometry described in the text. All values represent the averages of 8–12 determinations except for human livers which represent 2 samples.

study were obtained at local supermarkets and slaughterhouses and judged aflatoxin-free after assays by the method presented in this paper. Human liver was obtained from the Pathology Department of St. Francis Hospital, Peoria, IL. Standard aflatoxin solutions (0.1 ng B_1 and M_1/mL acetone) were added to blended beef liver (100 g/sample) to obtain samples spiked at levels of 0.1, 0.5, or 1.0 ng B_1 and M_1/g. The same solution was added to blended or ground swine, chicken, and human liver (100 g/sample), to beef kidney, spleen, heart, muscle (chuck), and blood, and to human urine (100 g or 100 mL/sample) at a level of 0.5 ng B_1 and M_1/g or mL. Both coagulated and uncoagulated (containing ethylenediaminetetraacetic acid (EDTA)) blood were tested.

Contaminated freeze-dried liver powder.—Contaminated dry powdered liver was prepared by thoroughly mixing standard aflatoxin solution (0.5 μg B_1 and M_1/mL acetonitrile) (3.6 mL) with aflatoxin-free blended liver (3.5 kg), and the mixture was freeze-dried. The dry powder was mixed 15 min with a Hobart mixer to ensure homogeneity. Analyses were made on samples equivalent to 100 g liver by mixing 30 g powder with 70 mL water in the extraction flask.

Extraction

Blend meat tissue until homogeneous (muscle and heart tissues must be ground because of insufficient water content). Weigh 100 g mixture or transfer 100 mL blood or urine into 500 mL wide-mouth, glass-stopper Erlenmeyer flask (or equivalent). Add 10 mL citric acid solution and mix thoroughly with 30 cm × 1 cm glass stirring rod. After 5 min, stir again, and mix with 20 g diatomaceous earth (40 g, blood; 50 g, urine). Add 200 mL dichloromethane and stir to remove excess solids from rod. Shake flask vigorously on wrist-action shaker (setting 5 on a Burrell) for 30 min. Filter mixture through paper (588-fast flow) into 300 mL Erlenmeyer flask containing 10 g sodium sulfate. (Close filter top and compress entire filter against funnel to obtain maximum filtrate volume.) Gently swirl flask intermittently ca 2 min and refilter through paper (560 or 2V-medium flow) (588 for blood) into 250 mL graduate and record volume (cover funnel with a watch glass to prevent evaporation of solvent). Evaporate filtrate in 500 mL round-bottom flask, under vacuum, to near dryness and save for column chromatography.

Column Chromatography

Proceed as in **26.A13** (11) except: (*1*) prepare column with dichloromethane; (*2*) redissolve concentrated filtrate in ca 25 mL dichloromethane and add to column (use dichloromethane to rinse round-bottom flask and column); and (*3*) use hexane–ether–acetonitrile (6 + 3 + 1) instead of (5 + 3 + 2).

Thin Layer Chromatography and Visual or Densitometric Analysis

Proceed as in **26.A14(c)** (11) for 2-dimensional TLC. Determine aflatoxin concentrations as in **26.074** and **26.031(d)** (11), substituting (100 g or mL × filtrate vol.)/200 in formula for *W*.

Results and Discussion

Recoveries of aflatoxins B_1 and M_1 from artificially contaminated meat tissues and blood are given in Table 1. Generally, recoveries for B_1

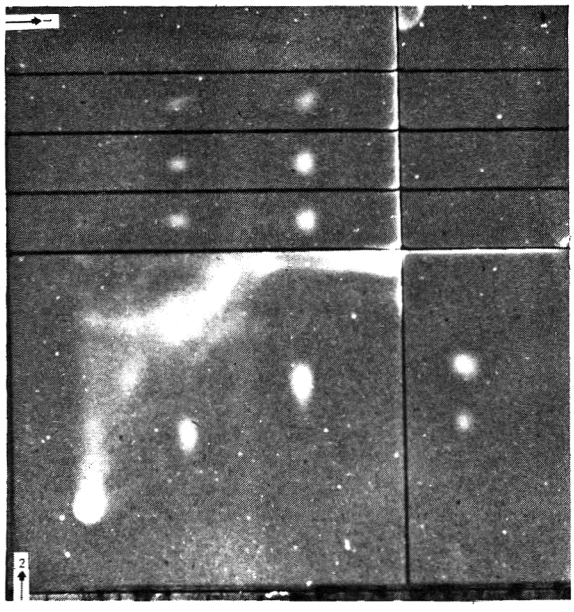

Figure 1. Photograph of a 10 × 10 cm TLC plate of beef liver extract containing aflatoxins B_1 and M_1 (0.5 ng/g each) developed 2-dimensionally with ether-methanol-water (93 + 6 + 1) (direction 1) and chloroform-acetone-isopropanol (82 + 10 + 8) (direction 2).

were 90–95% and those for M_1 were 80–85%. Coefficients of variation were 10–15% for B_1 and M_1 analyses. Only small differences were observed between levels (0.1–1.0 ng/g) in beef liver or between animal or tissue species. Recoveries from the 2 human livers tested were higher (104% B_1 and 97% M_1); however, data from only 2 samples were not representative. No unusual contaminants were observed on TLC plates for the human liver extracts. Two individual chicken livers (25–30 g each) were spiked with B_1 and M_1 (0.5 ng/g each), and the materials were reduced proportionately to determine if they could be assayed. Although the data are not given, individual chicken livers can be assayed successfully, but 4 livers (ca 100 g) are preferred. A comparison of the values for coagulated and uncoagulated beef blood indicated coagulated blood gave much better recoveries (95% B_1 and 93% M_1). This may be attributed to the swelling of red blood cells when blood is mixed with organic solvents, and either absorption, occlusion, or cleavage from bound proteins of the toxins during the extraction step. Another explanation may be that some aflatoxins form a complex with the EDTA used as an anticoagulant in the blood. The data on spiked samples indicated that coagulated blood is preferred for assay; however, this was difficult to predict without testing naturally contaminated blood samples.

The aflatoxin B_1 and M_1 zones on TLC plates were free of interfering substances for all samples. The cleanest extracts were obtained from blood samples. We occasionally found extraneous fluorescent zones near the B_1 zone in extracts of swine liver and near the M_1 zone in extracts of chicken liver; however, these impurities were not found routinely in these tissues. A typical 2-dimensional TLC plate of beef liver extract is shown in Figure 1.

A comparison of the assay results obtained with the 4 most recent analytical methods for liver (7–9) and for contaminated freeze-dried liver are presented in Table 2. The aflatoxin B_1 and M_1 values are very similar for all methods.

Table 2. Comparison of determination of aflatoxins B_1 and M_1 in contaminated powdered liver by 4 methods [a]

Method	Aflatoxin B_1, ng/g	Aflatoxin M_1, ng/g
Jemmali and Murthy (7)	0.42	0.48
Trucksess and Stoloff (8)	0.55	0.42
van Egmond et al. (9)	0.45	0.41
NRRC	0.43	0.40

[a] As determined by 2-dimensional TLC and densitometry. Contaminated powdered liver prepared by mixing aflatoxins B_1 and M_1 with blended beef liver, and then freeze-drying. All values are an average of 2 determinations made by H. P. van Egmond, National Institute of Public Health, Bilthoven, The Netherlands.

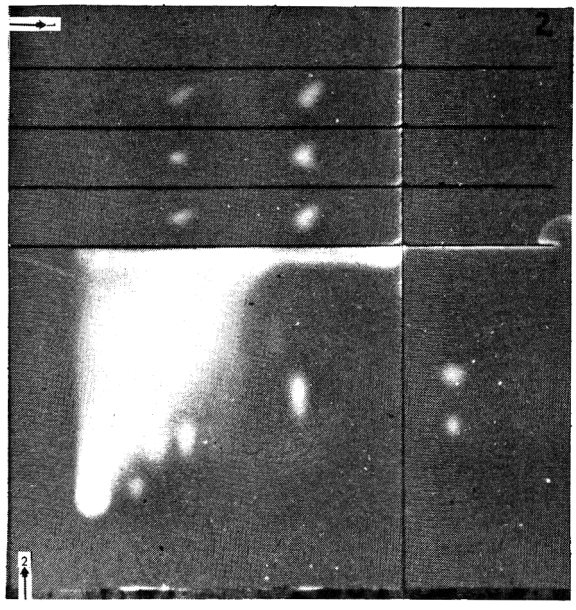

Figure 2. Photograph of a 10 × 10 cm TLC plate of contaminated freeze-dried beef liver (0.5 ng B_1 and M_1/g) developed as described in Figure 1.

The differences in the M_1 value attained by the Jemmali and Murthy method (7) and in the B_1 value from the Trucksess and Stoloff method (8) can be attributed to fluorescent interferences in those areas of the TLC plates. Extracts from the van Egmond et al. (9) and NRRC methods had less fluorescent impurities than did the extracts from the other methods. There are large differences in the total analysis times of the 4 methods. The methods of van Egmond et al. (9) and NRRC were faster and used less solvent and glassware. Overall for analyses of meats, the NRRC method compared favorably with the other methods.

These experiments showed a noticeable increase in interferences in the freeze-dried liver extracts which were not detected with fresh liver extracts (Figure 2). The freeze-drying process caused considerable changes in the meat tissue. This made interpretation of the TLC chromatograms for these products much more difficult—especially for the aflatoxin M_1. Assay of frozen meat products was definitely preferred over freeze-dried.

Several observations during the development of this method should be discussed. Most of the meat tissues could be blended to achieve homogeneity, but heart and other muscle tissues have lower water contents and, therefore, should be ground with a home food grinder. Trichloroacetic acid (10%) was investigated as a denaturing agent in place of citric acid, but this resulted in neither better recoveries nor cleaner TLC extracts. Dichloromethane was used throughout the method in place of chloroform, except in the eluting solvent for column chromatography. Dichloromethane is preferred because it is less toxic than chloroform and it does not contain ethanol as a preservative. The latter was important in the dairy products collaborative study (12) because ethanol content varied (0.75–2.0%) in commercial lots of chloroform throughout the world. Concentrations greater than 0.75% led to early elution of aflatoxin from the column. Dichloromethane eliminated this problem. Chloroform in the elution solvent (acetone–chloroform (1 + 4)) was not changed, because no problem would exist at this stage of the cleanup; however, higher ethanol concentrations would probably elute more interferences from the column. Presumably, dichloromethane could be substituted for chloroform in the eluting solvent if 0.75% ethanol was added to ensure a similar elution volume.

A medium flow filter was important to remove fine particulate matter from the concentrated meat extracts. Failure to do so would clog the silica gel column. Preferred filter papers are S&S No. 560 or Whatman No. 2V. The column cleanup step was modified from that originally proposed for the dairy products method (10) to include a 25 mL hexane wash to remove residual

acetic acid from the column. This was necessary to prevent early elution of B_1 and M_1 from the column.

Two-dimensional TLC of the meat extracts was accomplished with 10 × 10 cm plates and required only 30 min. This saved both time and cost. It should be mentioned that on TLC plates the aflatoxin B_1 and M_1 zones from the tissue extracts have slightly lower R_f values than the standards (Figure 1). This was caused by the large concentrations of extract that were spotted on the plate. The aliquot of extract spotted represented 12–15 g of original tissue, which was considerably more than usually spotted in other aflatoxin procedures (11). Toxin migration on plates during development in the first direction was slowed by the presence of impurities in the extract. This did not prevent positive identification of the aflatoxins, but one does need to become familiar with the position of the toxins on the TLC plate. False positive samples will not be a problem because confirmatory tests (13) should be run to positively identify any suspect samples.

Aflatoxins B_2, G_1, and/or G_2 can be detected in tissue samples with this method. For quantitative data on these aflatoxins, the solvent system that resolves them best should be substituted for the second-direction solvent (isopropanol–acetone–chloroform).

This method was tried unsuccessfully with 100 mL human urine samples. Cleanup of the samples was not satisfactory, and fluorescent contaminants obscured identification of B_1 and M_1 zones at levels less than 1 ng/mL. Acid and base partitions of the urine extracts were not effective in removing the contaminants. Animal urine samples were not available to the authors, so we did not try the method on these types of samples.

The method presented in this paper was effective for analyses of most meat tissues. It was rapid, gave clean extracts for TLC, used less toxic and smaller quantities of solvents and chemicals, and was sensitive to ≤0.1 ng/g.

Acknowledgment

The authors thank J. I. Greer (NRRC) and H. P. van Egmond (Institute for Public Health, Bilthoven, The Netherlands) for analyses of samples and E. A. Emken (NRRC) for procuring human liver samples.

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DRUGS IN FEEDS

High Pressure Liquid Chromatographic Determination of Nifursol in Finished Feeds

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Nifursol is extracted from feed with acetonitrile, and then washed with carbon disulfide to remove feed interferences. An aliquot is concentrated, diluted with 5% NaCl solution, filtered, and then subjected to high pressure liquid chromatography on a strong anion exchange column, using 365 nm photometric detection. The average recovery of nifursol from fortified feed samples was 100.6% with an average coefficient of variation of 3.5%. The nifursol determination is not subject to interference from the other commonly used nitrofurans.

Nifursol is a drug used to prevent blackhead (histomoniasis) in turkeys (1). It is administered through the feed at concentrations of 0.0025% (25 ppm)–0.0075% (75 ppm).

The present official AOAC method (**42.098–42.104**) for determining nifursol (3,5-dinitrosalicylic acid-(5-nitrofurfurylidene)hydrazide) in finished feeds involves extraction of the drug with dimethylformamide (DMF) (2, 3). Interferences are removed by alumina chromatography. The drug is detected and measured by reaction with phenylhydrazine hydrochloride to form the 5-nitrofurfural phenylhydrazone. The reaction product is partitioned into toluene and determined spectrophotometrically at 555 nm after addition of Hyamine ®-OH. This reaction relies on the response to the 5-nitro-2-furaldehyde (5NF) portion of the nifursol molecule, which makes the reaction subject to interference from other nitrofuran drugs.

A gas chromatographic procedure has been reported by Wheals and Weston (4) for determining nifursol in finished feeds. Nifursol is extracted from feed with acetonitrile, and the sample is washed with carbon disulfide to remove feed interferences. The nifursol molecule is hydrolyzed to form 3,5-dinitrosalicylic acid, which is then esterified with boron trifluoride–methanol complex to form methyl-3,5-dinitrosalicylate, which is detected and measured by electron capture gas chromatography. This method is sensitive and essentially specific for nifursol. The method, however, does require a specially prepared gas chromatographic column. Both of the above methods involve derivatization to determine nifursol in feed medication.

The use of high pressure liquid chromatography (HPLC) for determining the unchanged nifursol drug was investigated. This technique provided specificity without a derivatization step resulting in a convenient, accurate method for analysis of feeds for nifursol medication.

The HPLC method involves the extraction of nifursol from feed with acetonitrile followed by a carbon disulfide sample wash. The sample is concentrated by 50%, then diluted with a 5% NaCl solution, and fine-filtered. The sample is subjected to HPLC on a strong anion exchange column, using a 365 nm photometric detector. This analytical procedure for nifursol is not subject to interference from the other commonly used nitrofuran drugs.

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G. M. George is the Associate Referee for Nifursol in Feed.

METHOD

Reagents

(a) *Acetonitrile.*—Non-spectro, distilled in glass (Burdick & Jackson Laboratories, Inc.).

(b) *Carbon disulfide.*—Analytical reagent (Mallinckrodt Chemical Works).

(c) *Sodium chloride.*—Analytical reagent (J. T. Baker Chemical Co.). To prepare 5% solution, place 50 g sodium chloride in 1 L flask, and dissolve and dilute to volume with glass-distilled water.

(d) *Nifursol reference standard.*—3,5-Dinitrosalicylic acid-(5-nitrofurfurylidene)hydrazide (Salsbury Laboratories, Charles City, IA).

(e) *Stock standard.*—Place 50.0 mg reference standard in 100 mL volumetric flask. Dissolve and dilute to volume with acetonitrile. Prepare fresh daily.

(f) *Working standard.*—Dilute 3 mL above solution to 50 mL with acetonitrile. Use 5 mL of

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this solution to prepare HPLC standard. Prepare fresh daily.

Apparatus

(a) *High pressure liquid chromatograph.*—Equipped with Altex Model 153 detector set at 365 nm, range 0.02 absorbance unit full scale.

(b) *Recorder.*—Fisher Recordall Series 5000, 10 mV full scale; chart speed 0.2 in./min.

(c) *Sample valve.*—Rheodyne Model 7120, syringe-loading sample injector with 500 μL loop.

(d) *Pump.*—Milton Roy, capable of 5 mL/min and 5000 psi.

(e) *Column.*—2 ft × 1/8 in., stainless steel, with stainless steel end fittings. Pack with Dupont Zipax strong anion exchange (ZSAX).

(f) *Mobile phase.*—Mix 0.4% sodium perchlorate in water with equal volume of acetonitrile. Filter through 0.9 μm filter, and degas. Set flow rate at 3 mL/min.

Procedure

Grind feed sample ca 3 min in high speed blender to pass No. 20 sieve and mix thoroughly. Process feed sample as follows: Weigh 10.0 g feed sample into 100 mL volumetric flask. Add 50 mL acetonitrile and stopper flask with neoprene rubber stopper. Shake flask 45 min on Burrell wrist-action shaker. For pelleted feed, place sample in 70°C shaking water bath for 30 min followed by mechanical shaking for 15 min at room temperature. (Note: Allow contents in flask to warm to bath temperature before stoppering.) Remove and filter immediately by suction through a medium or fine sintered glass funnel.

Transfer 10 mL aliquot of filtered sample into 60 mL separatory funnel. Add 10 mL carbon disulfide and mix 30 s, and then let phases separate. Draw off lower (carbon disulfide) layer and discard. Transfer upper washed acetonitrile phase into 50 mL graduated conical centrifuge tube. Concentrate sample to 5.0 mL in a steam bath using gentle heating (70–80°C) and a filtered air stream to remove solvent vapors. Add 15.0 mL 5% NaCl solution to centrifuge tube and mix. At this point, include a standard by pipetting 5 mL aliquot of diluted standard into 50 mL conical centrifuge tube, plus 15 mL 5% NaCl solution. This standard is equivalent to 75 ppm nifursol (0.0075%) in 10 g of feed. Filter sample through fine sintered glass funnel. It is not necessary to filter standard. (Note: Prewash only the funnel with acetonitrile, and dry before sample is filtered.)

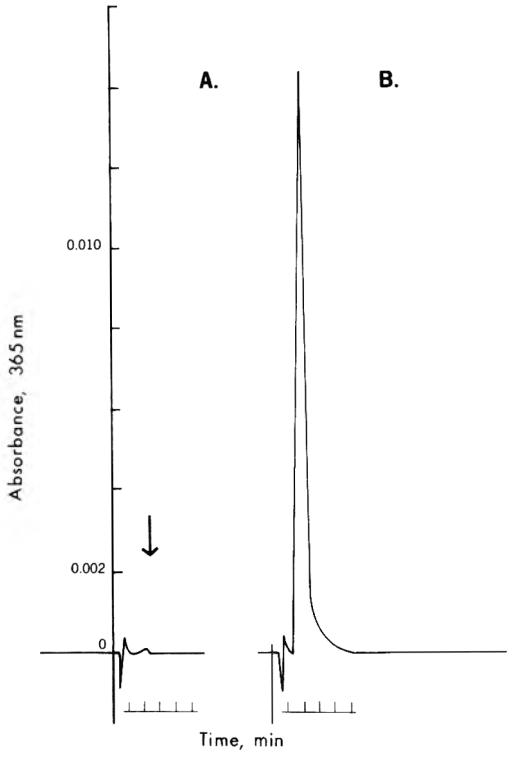

Figure 1. A. Chromatogram for control feeds; B. chromatogram for nifursol standard 75 ppm equivalent in feed.

Inject 500 μL sample into chromatograph. Approximately 8 min/chromatogram is required (see Figure 1 for typical chromatogram). Sample preparation time is approximately 2 h. Six samples can conveniently be prepared at a time. For best results, each day condition column with about 3 injections of sample before collecting data, and make standard injection after each sample injection.

Calculate nifursol as follows:

$$\text{nifursol (ppm)} = (\text{peak ht sample}/\text{peak ht std}) \times 75$$

Results and Discussion

The liquid chromatographic response to increasing amounts of nifursol was linear over the concentration range of 0–100 ppm nifursol in finished feeds. However, for higher concentrations of nifursol in feeds, a small sample should be extracted to keep the nifursol concentration in this linear response range. The concentration of nifursol is quantitated from a single point standard. It is unnecessary to es-

Table 1. Recovery of nifursol from laboratory-medicated feeds

	Fortification level, ppm nifursol			
Sample	25	50	75	100
1	27	54	73	103
2	27	54	71	101
3	24	48	73	97
4	24	48	72	95
5	26	47	77	95
6	26	51	77	94
7	27	49	75	101
8	27	54	76	99
9	25	53	73	98
10	25	50	72	100
11	26	50	72	99
12	25	50	75	101
13	27	49	74	99
14	26	53	77	98
15	27	54	77	100
16	26	53	75	99
17	27	52	74	93
18	26	49	72	93
19	25	52	72	97
20	25	51	—	—
21	—	48	—	—
Mean	25.9	50.9	74.1	98.1
SD	1.03	2.32	2.04	2.73
CV, %	3.9	4.6	2.8	2.8
Rec. %	103.8	101.8	98.8	98.1

tablish a standard curve daily, because linear response has been established over this defined concentration range.

Recovery of nifursol from laboratory-medicated feeds was studied over the range of 25–100 ppm (Table 1). A nonmedicated commercial formulation of turkey starter mash was fortified in the laboratory with 25, 50, 75, or 100 ppm nifursol. The medicated feed samples were then sampled and subjected to HPLC analysis by the described procedure. The average observed recovery for 20 feed samples fortified at 25 ppm was 103.8%, with a coefficient of variation of 3.9%. The average observed recovery for 21 feed samples fortified at 50 ppm was 101.8% with a coefficient of variation of 4.6%. The average observed recovery for 19 feed samples fortified at 75 ppm was 98.8% with a coefficient of variation of 2.8%, and the average observed recovery for 19 feed samples fortified at 100 ppm was 98.1% with a coefficient of variation of 2.8%. The method is accurate with an overall average recovery of 100.6% and precise with an average coefficient of variation of 3.5%.

Sixteen different nonmedicated feed samples (1–8 mash, 9–16 pellets) were analyzed by the described assay procedure to determine the typical nonmedicated feed response calculated as nifursol: 0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 0.5, 0.5, 0.3, 0.5, 0.7, 0.5, 0.5, 0.5, 0.5, 0.5. The average nifursol response of these nonmedicated feeds was 0.3 ppm, range 0.0–0.7 ppm. These feed samples involved starter-, grower-, and finisher-type rations so that a variety of feed formulations was represented.

The following feed additives were assayed by the described nifursol procedure for possible interferences: nitrofurazone, nihydrazone, furazolidone, and nicarbazin. None interfered with the assay. The compounds emerge from the column at the solvent front. Retention time for nifursol is 2 min. Nicarbazin has a very short retention time, but is separated from nifursol. The compound is insoluble in acetonitrile, so a very small peak is observed. All the above drugs were checked at the 100 ppm feed medication level. To check for interference, actual feed samples were fortified with the drugs in question and were assayed as described.

During the method research work, another workable liquid chromatographic column packing material was tried. A 1 m × 4.6 mm diameter column packed with Bondapak C_{18} reverse phase (37–50 μm particles (Waters Associates)) provided good results for nifursol. However, nitrofurazone interfered, producing a peak on the tail of the nifursol peak. A good separation of nifursol and nitrofurazone could not be achieved by adding more column length and varying the conditions. Reverse phase μBondapak C_{18} has been used for determining nitrofurantoin in urine and plasma (5) and furazolidone in turkey tissue (6).

Nifursol in poultry rations was assayed in the presence of several other animal health products to determine whether they might interfere with the nifursol assay (H. Knapstein (1978) Kiel Agricultural Research Center, personal communication). These products included: Zoalene, nitrofurazone, nicarbazin, carbadox, Furoxone, Nitrovin, Coyden, ipronidazole, dimetridazole, decoquinate, buquinolate, nequinate, ronidazole, amprolium, robenidine, ethopabate, pyrimethamine, thiabendazole, diaveridine, and olaquindox. No interference either positive or negative was observed from these products.

The best detection for nifursol is observed in the visible region. The peak maximum for nifursol in acetonitrile–NaCl solution is 375 nm. A 375 nm filter was not available for the Altex detector so a 365 nm filter was used with good results. The fact that the feed extract blank shows no peaks to interfere with nifursol also indicates that the 365 nm wavelength is acceptable. Au-

Table 2. Assay of several samples of commercial feeds by the proposed HPLC procedure and official AOAC method

	Nifursol obsd, ppm	
Sample ident.	AOAC method	Proposed HPLC method
8109	24	25
8110	67	68
8578	6.1	0.0
1974	53	58
1327-R	67	55
	61	62
		59
1328-R	62	58
		58
1329-R	72	59
	73	53
	68	52
	74	
1330-R	59	52
1331-R	67	48
	60	50
	65	53
	62	
1332-R	2.0	0.4
1333-R	35	34
1334-R	15	14
1335-R	5.0	0.4

frere et al. (5) also report a 370 nm absorption maximum for nitrofurans. Response in the UV region is rather poor at both the 254- and 280-nm wavelengths.

Should the retention time diminish on the Zipax column through use, the retention and/or separation for nifursol can be restored by reducing the percent sodium perchlorate in the aqueous portion of the mobile phase.

It is recommended that the ZSAX column be stored overnight in acetonitrile–water (1 + 1). Prolonged storage of the column in water alone may result in bacterial growth which will clog the sintered metal filter disks in the column end fittings. If methanol–water is used for overnight storage of the column, the methanol will suppress nifursol response on subsequent chromatograms. The acetonitrile–water storage of the column eliminates this difficulty.

Several samples of commercial pelleted feeds were assayed by the proposed HPLC procedure and by the present official AOAC method (2, 3) for nifursol content. Since these were commercial feeds, the true amount of nifursol in the feed is not known. The feeds were analyzed to compare results obtained by the HPLC procedure to the official AOAC procedure; results are reported in Table 2. Generally, the 2 methods produce quite similar results. For pelleted feeds it was necessary to extract with acetonitrile in a 70°C shaking water bath for 30 min to remove the nifursol. This was not necessary for mash-type feeds.

For 2 feed samples (1329-R and 1331-R), the 2 methods show some difference in results. The AOAC method results are approximately 20–25% higher for nifursol in these 2 feeds when compared with the HPLC procedure. A portion of the AOAC extract was subjected to the HPLC detection. The HPLC detection did not find any more nifursol in the AOAC extract than in the acetonitrile extract. It was concluded from this test that for feeds 1329-R and 1331-R, the AOAC method produced erroneous results, presumably due to a positive interference in the rather nonspecific AOAC colorimetric method. It was noted on the chromatograms for 1329-R and 1331-R feeds that something eluted very quickly with little or no retention on the column. This was not observed on the other feed samples analyzed by HPLC.

From results generated in the HPLC method evaluation, we conclude that a suitable HPLC method has been developed for nifursol in animal feeds. The method is both accurate (100.6% recovery) and precise (3.5% CV). The method is specific for nifursol; other commonly used nitrofurans and feed additives do not interfere.

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Liquid Chromatographic Method for Determination of Arprinocid in Feed: Collaborative Study

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An HPLC method for determining arprinocid [9-(2-chloro-6-fluorophenyl)methyl-9*H*-purin-6-amine] in feed was evaluated in an interlaboratory collaborative study. The samples were prepared in pairs from feeds obtained from 2 commercial feed manufacturers to cover the concentration range 0.0050–0.0070% arprinocid and were distributed to 14 laboratories. Each collaborator was requested to perform one determination on each of 6 samples. In this analytical procedure, the drug is extracted into $CHCl_3$ and, after appropriate sample preparation by liquid-liquid partitioning, determined using a silica column and ultraviolet detection. The means of the analyses reported by the collaborators ranged from 97 to 104% of the true concentration of arprinocid and were not significantly different ($P > 0.1$) from the true values. The average coefficient of variation was 6.8%. The precision standard deviations of the 3 unit blocks (s_r) were each <0.0004% arprinocid, and the *F*-test demonstrated that systematic error (s_b) did not make a statistically significant contribution ($P > 0.1$) to the standard deviation of the data (s_d). This method has been adopted official first action.

Arprinocid [9-(2-chloro-6-fluorophenyl)-methyl-9*H*-purin-6-amine] (Figure 1), a new anticoccidial agent, can be determined in feed in the concentration range 0.0045–0.0080% by either a colorimetric (1) or a high pressure liquid chromatographic (2) analytical procedure. The latter (HPLC) method was developed to provide a much faster and more rugged method than the colorimetric procedure. For this reason, and also because HPLC is being used increasingly in the analysis of animal feeds, the chromatographic method was selected for collaborative study.

Collaborative Study

Preparation and Distribution of Collaborative Samples.—The 3 statistical unit blocks were prepared at concentrations of 0.0050, 0.0060, and 0.0070% arprinocid to cover a range of concentrations ±0.0010% of the expected finished feed concentration of 0.0060%. The 2 samples of each unit block were formulated by blending the same weight of a drug premix into 2 unmedicated commercial broiler mash feeds. A single premix, containing 12% arprinocid, was used to prepare each of the 6 samples. Before it was blended into the feeds, the premix was analyzed several times: 6 replicate analyses yielded 100% recovery, confirming the 12% arprinocid premix concentration.

One hundred pound batches of each broiler mash were mixed with the appropriate weight (ca 19–27 g) of the 12% premix for each feed concentration level in a 3 cu. ft ribbon mixer. Each blend of mash was permitted to mix for 20 min to ensure homogeneous distribution of arprinocid. The medicated mashes were then sampled and analyzed, and the balance was converted to pellets: A steam pressure of about 10 psi increased the conditioning temperature to about 170°F. The final 25 lb of pellets was spread in a thin layer to facilitate cooling and drying. The pellets were air-dried 16 h. Final arprinocid concentration level and homogeneity were confirmed by replicate analyses of each collaborative sample.

The 6 feed samples which were circulated to 14 collaborators were randomly numbered as follows. The arprinocid concentration in consecutive sample number order was: Sample 1—0.0060%, Sample 2—0.0070%, Sample 3—0.0050%, Sample 4—0.0050%, Sample 5—0.0070%, Sample 6—0.0060%. Collaborative Samples 1, 2, and 3 were prepared by medicating commercial feed formula A, and Samples 4, 5, and 6 represented feed B. Each collaborator was supplied with 100–125 g of each feed sample, 0.5 g analytical reference standard arprinocid, a copy of the method, and a report form. The collaborators were told only that all samples contained arprinoced in the concentration range 0.0040–0.0080%. Each collaborator was requested to perform a single analysis on each feed, although duplicate injections of the final analytical solu-

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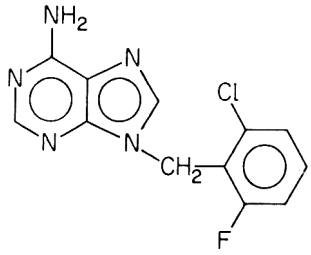

Figure 1. Arprinocid.

tions were suggested as a check on the instrumentation. Each laboratory was also requested to submit representative chromatograms with the report form.

Pretest of the Method.—In advance of distributing the collaborative samples, the method was evaluated in a preliminary internal collaborative study carried out by different analytical chemists within the Associate Referee's laboratory. Two carefully blended medicated mash feeds were examined—one sample contained 0.0060% and the other contained 0.0070%. These samples were each analyzed 6–7 times by 3 analytical chemists. The results showed that there was no statistically significant difference among the results obtained by the 3 chemists; with the exception of only 1 outlying result, each of the 54 determinations performed was within the range ±0.0005% of the labeled medicated drug concentration. The overall average recovery was 102% of the label claim for 34 analyses at the 0.0060% concentration level and 100% of the nominal concentration for 20 analyses of the 0.0070% medicated feed. The coefficients of variation at these 2 concentration levels were 3.3% and 4.0%, respectively, under these conditions.

Arprinocid

High Pressure Liquid Chromatographic Method – Official First Action

(*Caution:* See **51.005, 51.011, 51.018, 51.056, 51.061**)

42.B01 ***Principle***

Arprinocid is extd from feed into $CHCl_3$ in presence of pH 7 phosphate buffer and sepd from interferences by partitioning between hexane and 0.1N HCl. Aq. soln is neutzd and drug is extd into $CHCl_3$ for direct measurement by adsorption HPLC on silica column with photometric detection at 254 nm.

42.B02 ***Reagents and Apparatus***

(a) *High pressure liquid chromatograph.*—Model 740B pump and Model 8200 photometric detector (Spectra-Physics, Inc., Mountain View, CA 94040), or equiv. Operating conditions: flow rate 1.0 mL/min; detector wavelength 254 nm; 100 μL loop injection valve (Valco Instruments Co., Inc., Houston, TX 77055); ambient temperature.

(b) *Chromatographic column.*—25 cm × 3.0 mm id, contg Spherisorb 5 μm silica (Spectra-Physics, Inc.).

(c) *Mobile phase.*—MeOH-H_2O-$CHCl_3$ (3 + 0.2 + 97). ($CHCl_3$ should contain ca 1% alcohol as stabilizer.)

(d) *Phosphate buffer.*—0.1M. Dissolve 6.80 g KH_2PO_4 and 8.71 g K_2HPO_4 in 1 L H_2O.

(e) *Arprinocid std soln.*—24 μg/mL. Weigh 60.0 mg arprinocid and dissolve in 250 mL $CHCl_3$. Evap. 5.00 mL aliquot to dryness under N in 50 mL vol. flask; dissolve residue in and dil. to vol. with 0.1N HCl.

42.B03 ***Extraction***

Accurately weigh 10.0 g ground feed and transfer to 125 mL g-s erlenmeyer. Add 50.0 mL $CHCl_3$ and 25.0 mL 0.1M phosphate buffer, and mech. shake 1 h. Transfer ca 45 mL extn mixt. to 50 mL centrif. tube. Centrif. 10 min at 2000–2500 rpm and discard upper (aq.) phase. Filter $CHCl_3$ phase thru Whatman No. 2 paper and transfer 10.0 mL filtrate to 15 mL centrif. tube. Place tubes in ca 50° H_2O bath and evap. under N to ca 0.25 mL oily residue.

42.B04 ***Partitioning***

Add 5.0 mL hexane followed by 5.00 mL 0.1N HCl to tube contg residue and mech. shake 10 min at low speed (to avoid formation of emulsion). Centrif. 10 min at 2000–2500 rpm and discard upper (hexane) layer and any emulsion at interface. Transfer 3.00 mL aq. sample soln to 15 mL centrif. tube and add 3.0 mL 0.2M Na_2CO_3 and 3.00 mL $CHCl_3$ to tube. Transfer 10.00 mL arprinocid std soln to 50 mL centrif. tube and add 10.0 mL 0.2M Na_2CO_3 and 10.00 mL $CHCl_3$ to tube. Mech. shake both sample and std 10 min and centrif. at 2000–2500 rpm. Discard upper (aq.) phase.

42.B05 ***Determination***

Use $CHCl_3$ solns of sample and std for HPLC injections. Using 100 μL loop injection valve inject std, follow with duplicate injections of sample, and repeat injection of std. Measure peak hts, obtain mean peak hts for sample (*PH*)

Table 1. Collaborative results for the determination of arprinocid (%) in feed

	Arprinocid found, %					
Coll.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	0.0063	0.0067	0.0053	0.0056	0.0076	0.0066
2	0.0064	0.0072	0.0050	0.0052	0.0070	0.0063
3	0.0063	0.0070	0.0048	0.0054	0.0072	0.0061
4	0.0065	0.0068	0.0050	0.0053	0.0073	0.0068
5	0.0059	0.0071	0.0048	0.0050	0.0072	0.0059
6	0.0060	0.0066	0.0052	0.0050	0.0066	0.0057
7	0.0084[a]	0.0069	0.0052	0.0050	0.0075	0.0041[a]
8	0.0051	0.0069	0.0039[a]	0.0057	0.0057	0.0065
9	0.0072[a]	0.0077[a]	0.0063[a]	0.0058[a]	0.0081[a]	0.0073[a]
10	0.0051	0.0060	0.0046	0.0052	0.0061	0.0054
11	0.0062	0.0072	0.0052	0.0052	0.0074	0.0068
12	0.0050	0.0062	0.0051	0.0047	0.0071	0.0059
13	0.0060	0.0069	0.0048	0.0051	0.0069	0.0060
14	0.0068	0.0070	0.0055	0.0055	0.0072	0.0062
Average, %	0.0060	0.0068	0.0050	0.0052	0.0070	0.0062
CV, %	10	5.3	5.1	5.3	7.9	7.1
Formulated concn, %	0.0060	0.0070	0.0050	0.0050	0.0070	0.0060
Arprinocid rec., %	100	97	100	104	100	103

[a] Outlying results excluded from statistics.

and standard (*PH'*), and det. arprinocid with formula:

$$\text{Arprinocid, \%} = (PH/PH') \times 0.0060$$

Results and Discussion

The results of the collaborative study are presented in Table 1, for which the data from some laboratories were rounded to 2 significant figures to reduce all the results to a consistent set. This table shows that the means of the recoveries of arprinocid on each sample ranged from 97 to 104%. The following statistical treatment is in accord with the guidelines of the *Statistical Manual of the AOAC* (3).

The 2-sample charts for the 3 unit blocks are presented in Figure 2. As usual, the points are predominantly found in quadrants I and III, respresenting +,+ and −,− results: 20 points lie in these 2 quadrants, 13 appear in quadrants II and IV, and the remainder of the laboratories are located on the borders between quadrants. The points are also generally clustered near the reference concentrations in the usual elliptical pattern around a major axis at 45°, with a few apparent exceptions: The 3 points for collaborator 9 all lie well out of the cluster, although near the 45° line for each pair. This pattern is clear evidence of a systematic error which is large in comparison with the other collaborators. Collaborator 7 is also far removed from the cluster on pair II, and Collaborator 8 shows evidence of an atypically large precision error.

In general, most of the representative chromatograms submitted by the collaborators were of excellent quality: The shape of the analyte peaks indicated high chromatographic efficiency and were well separated with baseline resolution from all extraneous feed components at short retention times. Because Collaborators 4 and 6 found the arprinocid peaks on the tail of the elution of other components, these collaborators drew sloping baselines for their peak height measurements; this procedure did not have a deleterious effect on the analytical results of these laboratories (Table 1).

Rejection of Outliers.—The results of ranking the collaborative analyses are presented in Table 2. Collaborator 9 reported the highest analytical results on 5 of the 6 samples, and also ranked No. 2 on the remaining feed, for which the highest result proved to be an individual outlier (see below). The total rank for this laboratory, 7, is well beyond the approximate 5% 2-tail limits for ranking 14 collaborators with 6 samples, which are 17 and 73. With this ranking, which is also apparent in the 2-sample charts, Laboratory 9 was not included in the further analysis of the data because of this abnormally large systematic error.

The total rank for Laboratory 10 is also beyond the criteria for rejecting an outlying laboratory.

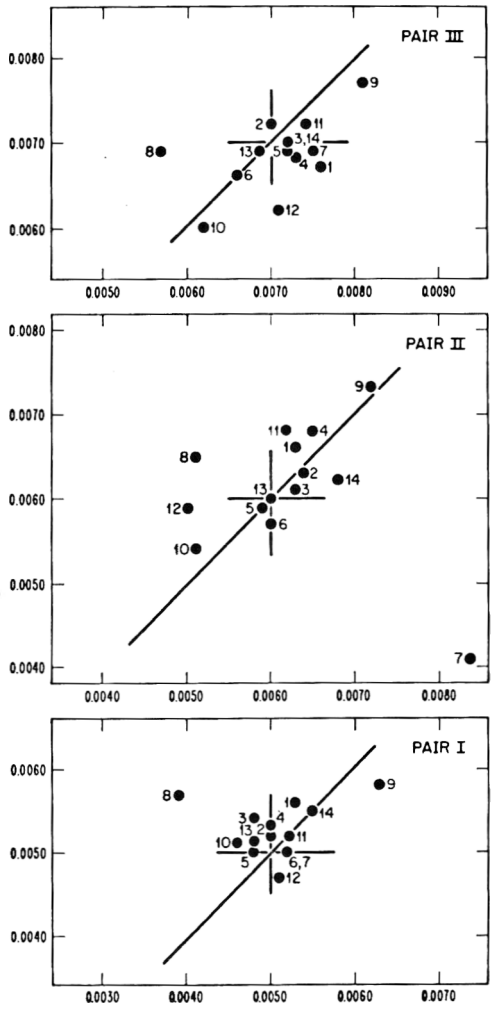

Figure 2. Two-sample charts for the 3 statistical unit blocks. Each pair consists of the same concentration of arprinocid blended into 2 different commercial feed formulas. The numbers beside the points identify the collaborators. The reference values are indicated by the cross-hatch at the center of each chart.

However, the rank for this collaborator generally reporting low results is only 0.5 unit beyond the statistical limit. In addition, the points for this collaborator are not consistent among the three 2-sample charts: Although this laboratory appears in the third quadrant (−,−) on pairs II and III, it can also be found in the second quadrant (−,+) on pair I. For these reasons, Collaborator 10 was retained in the following statistical analysis.

There are 3 individual laboratory results which are beyond the limits established by the normal law of errors as evaluated by Dixon's test. These are the results of Laboratory 7 for Samples 1 and 6 and of Laboratory 8 for Sample 3. It is significant that 2 of these 3 outlying values were reported by one collaborator. Calculated values of r_{21} for these 3 values are 0.58, 0.64, and 0.64, respectively, and the critical value of r_{21} for 13 measurements is 0.52. Hence, because each of these ratios is beyond the criterion for rejection with a 1 in 20 probability of a wrong decision, these 3 individual outlying results were excluded.

Homogeneity of Variation between Laboratories.—The homogeneity of experimental variation between laboratories was evaluated based on the ranges of results on the collaborative samples. After elimination of the outliers, the ranges, ω_k, of Samples 1-6 are: 0.0018%, 0.0012%, 0.0009%, 0.0010%, 0.0019%, and 0.0014%, respectively, yielding a ratio $\omega_{max}/\omega_{min}$ = 0.0019/0.0009 = 2.1. This ratio is below the critical limit for testing homogeneity of within-sample variation at the 5% level of significance (2.7). Thus, the variance is demonstrated to be sufficiently homogeneous so that none of the samples need be treated separately.

Statistical Results.—Table 3 summarizes, in standard form, the results of the statistical study. The precision standard deviation, s_r, was computed from the differences of the paired samples, the distribution standard deviation, s_d, from the totals of the pairs, and the standard deviation for the distribution of systematic errors, s_b, from the relationship $s_d^2 = 2s_b^2 + s_r^2$. In all 3 pairs, s_r and s_b are each <0.0004% arprinocid and are also of the same order of magnitude, demonstrating that neither of these sources of error is unusually dominant. The calculated F-values in Table 3 are less than the critical F at the 10% level of significance for 12 degrees of freedom; hence, systematic error, s_b, does not make a statistically significant contribution ($P > 0.1$) to the standard deviation of the data, s_d. The coefficients of variation listed in Table 3 were computed in a standard manner from all of the individual analyses at each concentration level. Because these coefficients are independent of concentration, the mean of these 3 values, 6.9%, is an accurate measure of the precision of the analytical method.

Accuracy.—Because the true reference concentration of arprinocid is known in this collaborative study for each unit cell, the Student t-test can be used to test for systematic error in this method. For pairs I, II, and III, the calculated values of t are 1.7, 0.87, and 0.95; for corre-

Table 2. Ranked collaborator results

Coll.	Sample 1	2	3	4	5	6	Total rank
1	6.5	11	3	3	2	4	29.5
2	5	2.5	8.5	8	10	6	40
3	6.5	5.5	11	5	7	8	43
4	4	10	8.5	6	5	2.5	36
5	11	4	11	12	7	10.5	55.5
6	9.5	12	5	12	12	12	62.5
7	1	8	5	12	3	14	43
8	12.5	8	14	2	14	5	55.5
9	2	1	1	1	1	1	7
10	12.5	14	13	8	13	13	73.5
11	8	2.5	5	8	4	2.5	30
12	14	13	7	14	9	10.5	67.5
13	9.5	8	11	10	11	9	58.5
14	3	5.5	2	4	7	7	28.5

sponding s_T values of 0.00040, 0.00080, and 0.00076%, respectively. These t-values are less than the appropriate critical t at the 10% level of significance, demonstrating that there is no statistically significant difference ($P > 0.1$) between the average analytical results and the true (reference) values.

Effect of Chromatographic Column.—This HPLC procedure affords a unique opportunity among collaborative studies to examine the effects of different instrumentation, particularly the selection of analytical chromatographic columns by different collaborators. The results on the 6 different columns which were sampled in this collaborative study are grouped according to column in Table 4. Although no statistical analysis was carried out because of the unequal numbers of laboratories which used each column, these results demonstrate that there are several commercially available chromatographic columns which are satisfactory for this analysis. The 91% recovery on Zorbax Sil cannot be assigned to column effects because this column was used by only one collaborator.

Application to Premix.—The drug premix which is used for medicating feeds contains 12% arprinocid and is added to feed in the proportions of 1 lb premix per ton feed to yield a final finished feed concentration of 0.0060% arprinocid. Accordingly, the general applicability of this method to the analysis of arprinocid premixes was evaluated by performing 7 replicate analyses on each of 3 medicated premixes: These premixes were formulated to contain 10.2, 12.2, and 14.3% arprinocid to cover a range of concentrations around the proposed use level. In this modification of the feed method, 0.6250 g premix was extracted for 1 h into 250 mL $CHCl_3$, and 10.0 mL filtered extract was diluted to 100 mL with $CHCl_3$ before injection into the HPLC system. The results, presented in Table 5, illustrate the satisfactory analysis of the premix by this modification of the feed procedure. All of these premix analyses were obtained by one analytical chemist on one day.

Comments of Collaborators

Two of the collaborators commented that they felt that more precise and accurate results can be obtained when the analytical chemists have more experience with the procedure. Extensive experience in the Associate Referee's laboratory confirms this prediction. It should be noted that a practice sample was not sent out with these test feeds, so that the results presented in Table 1 represent the first experience of each laboratory with the procedure. The samples were numbered in consecutive order and each laboratory

Table 3. Results of statistical analysis of the collaborative results for arprinocid

Pair	Arprinocid present, %	Av. arprinocid found, %	Diff. %	Precision s_r	Systematic s_b	Distribution s_d	F s_d^2/s_r^2	DF	CV %	Arprinocid rec., %
I	0.0050	0.0051	0.0001	0.00022	0.00012	0.00028	1.6	11	5.4	102
II	0.0060	0.0061	0.0001	0.00039	0.00029	0.00057	2.1	11	8.6	102
III	0.0070	0.0069	0.0001	0.00038	0.00027	0.00054	2.0	12	6.8	99

Table 4. Effect of chromatographic column on collaborative results (% recovery of arprinocid)

Chromatographic column		No. of coll.	Coll.	Sample 1	2	3	4	5	6	Av.
Waters	μPorasil Silica	4	1, 2, 3, 6	104	98	102	106	101	103	102
Waters	Radial Compression Radial Pak B	1	5	98	101	96	100	103	98	99
Perkin-Elmer	Silica A	2	4, 7	108	98	102	104	106	113	105
Spectra-Physics	Silica 5	4	11, 12, 13, 14	100	98	103	103	102	104	102
Hibar II	LiChrosorb S160	1	8	85	99	—	114	81	108	97
DuPont	Zorbax Sil	1	10	85	86	92	104	87	90	91

analyzed them in sequence. Hence, that the coefficient of variation on Sample 1, 10%, is greater than that of any of the later samples is a clear indication of improved results with experience. Two of the collaborators reported second trials on their samples for the following reasons: Collaborator 7 did not follow the method as written the first time because he was unable to grind the samples. Collaborator 5 repeated his analyses because of his evaluation of the performance of the instrumental chromatographic system during the first run. Even after the second run, one of his results by integrated peak area differed by 7% from the same chromatogram calculated using peak height measurements. The Associate Referee selected this collaborator's results by peak height measurement for inclusion in the statistical analysis to avoid possible instrumental integrator errors and to conform to the written procedure.

Collaborator 1 analyzed 2 trial unmedicated feed spikes to check the chromatographic conditions before analyzing the test samples: the recoveries at the 0.004 and 0.008% arprinocid concentration levels were 109 and 99%, respectively. Two collaborators experienced difficulty in collecting 10.00 mL $CHCl_3$ extract after filtration, and corrected their calculation for a smaller volume taken at this step. An emulsion at the interface of this partition was also noted. One collaborator reported reproducibility problems with his autosampler, and Collaborator 12 had difficulty dissolving the analytical reference standard in 0.1N HCl. Several collaborators used minor modifications of the mobile phase which helped to optimize their chromatographic systems. We have been unable to account for the extraordinarily precise high systematic error of Laboratory 9: The Associate Referee analyzed the 24 μg/mL standard solution used by this collaborator and found that it was accurately prepared (99–101%). It is possible, similar to Collaborator 12, that this collaborator perhaps did not achieve quantitative dissolution of the arprinocid standard in the 0.1N HCl.

Table 5. Application of HPLC procedure to arprinocid premixes

Replicate	Premix concentration (% arprinocid) 10.2	12.2	14.3
1	10.2	12.0	14.3
2	10.2	11.9	14.2
3	10.2	12.0	14.3
4	10.3	11.9	14.3
5	10.3	11.9	14.2
6	10.3	11.9	14.4
7	10.3	11.8	14.4
Average	10.3	11.9	14.3
Rec., %	101	98	100
Range, %	0.1	0.2	0.2

Conclusions and Recommendation

In addition to these collaborative feed samples, this HPLC procedure has also been evaluated with satisfactory results in more than 20 different feeds of both domestic and foreign origin. The accuracy of the method has also been confirmed by correlation with 2 other independent methods. A feed prepared to contain 0.0070% arprinocid was analyzed by the present procedure and also by a method using the same chromatographic measurement after extraction into acetone rather than $CHCl_3$. (The solubility of arprinocid in acetone is 1.0 mg/mL.) The 13 analyses using the collaborative method averaged 0.00704% arprinocid with a coefficient of variation of 4.6%, while the average of 7 analyses following acetone extraction was 0.00701% with a 2.0% coefficient of variation. Previously, agreement was also good between this method and an independent colorimetric analysis which uses the same $CHCl_3$ extraction technique (2). The former correlation supports the efficiency of the extraction procedure and the latter con-

firms the accuracy of the measurement operation of this collaborative method. Prior reports have also confirmed the extraction efficiency by radiotracer counts of the $CHCl_3$ extract from a feed blended with labeled arprinocid (1), and demonstrated that this method is applicable to aged as well as to fresh feeds, and to both mash and pellet feed forms (2).

It has been pointed out by a collaborator that it would be helpful to have an alternative method based on reverse phase HPLC in addition to the normal phase method available for any laboratory. The Associate Referee agrees that this would be useful and recommends continued study of arprinocid with this objective. Based on these collaborative results, it is recommended that the present method be adopted official first action. Finally, it was noted earlier that the statistical analysis was performed according to the *Statistical Manual of the AOAC* after elimination of 1 outlying laboratory and 3 outlying individual results. Inclusion of all the collaborative data of Table 1 in the calculations, however, does not markedly change the results, i.e., with all the results included, the recoveries on the 6 collaborative samples averaged over all 14 collaborators are: 103, 99, 102, 106, 101, and 102%, for Samples 1-6, respectively, yielding an average recovery of 102%.

Note Added in Proof: After completion of the collaborative study, outlying Laboratory 9 provided repeat analyses on 3 of the collaborative samples. The analytical results on this second trial of the method—0.0063, 0.0072, and 0.0053% on Samples 1-3, respectively (*i.e.*, recoveries of 105, 103, and 106%)—reflect improved results with experience with the method.

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Bacitracin Determination in Feeds: Evaluation of Methods

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Assay of bacitracin activity in feeds is subject to many sources of variation. Bacitracin standards are complex mixtures and may contain various amounts of less active forms of bacitracin. Test organisms may respond differently to these mixtures. Collaborative studies should involve the use of one standard, perhaps USP zinc bacitracin. The use of different types of bacitracin standards (regular or zinc) in a laboratory may contribute to variation in sample potency. The pyridine extraction method is subject to serious operational difficulties. Among the contributing factors are incomplete evaporation of pyridine (causing positive bias), allowing the temperature to increase too much during evaporation (causing negative bias), and the numerous manipulative steps in the technique (causing large variability). Because of these factors and the toxic properties of pyridine, the method finds little use. Methanol extraction offers a better substitute for determining zinc or methylene disalicylate bacitracin in premixes and complete feeds by the plate method.

Bacitracin was first described by Johnson et al. (1) as an antibiotic produced by a strain (Tracy-I) of *Bacillus subtilis* later classified as *B. lichenformis*. It was isolated from a contaminated wound. Studies by Craig et al. (2) in the United States and Abraham (3) and Newton and Abraham (4) in England showed the antibiotic to be a mixture of closely related polypeptides. The chief, and most biologically active, component is bacitracin A (3, 5). Other components such as bacitracins B, D, E, and F (2, 6) are present in smaller concentrations, they are less active, and their structural formulas are not well defined. The molecular weight of bacitracin A is approximately 1411. It contains several amino acid residues such as L-cysteine, D-ornithine, L-lysine, L-histidine, D-aspartic acid, D-glutamic acid, L-isoleucine, L-leucine, and D-phenyl alanine (7). In neutral or slightly alkaline solutions, bacitracin A is slowly transformed to bacitracin F (8). Bacitracin from different commercial sources contains different proportions of the component bacitracins.

Bacitracin is a white, amorphous, hygroscopic powder. It is precipitated from aqueous solutions by zinc, methylene disalicylate, and manganese salt. Not all findings regarding solubility of bacitracin salts are identical.

Zinc bacitracin contains about 7% zinc. It is relatively insoluble in petroleum ether (0.025 mg/mL) but readily soluble in acid solution (5.1 mg/mL), in methanol (6.55 mg/mL), in pyridine (4.05 mg/mL), and in formamide (>20 mg/mL) (9). In 1957, a U.S. patent was issued to Commercial Solvents Corp. for their method for commercial production (10).

In 1956, the commercial process for production of methylene disalicylate (MD) bacitracin was patented in the United States by S. B. Penick and Co. (11). The sodium or potassium salts of MD bacitracin are soluble in water, in sodium carbonate solution, or in alkalies between pH 7.0 and 8.5. The acid form is insoluble in water and in the usual organic solvents.

Numerous observations (12–14) have been made on the effect of metal binding on bacitracin activity. In general, zinc, cobalt, manganese, and calcium enhance bacitracin activity, whereas copper interferes (causes negative bias) with the assay (6, 15).

Bacitracin has been added to animal feed for growth promotion, feed efficiency, and disease control. The zinc and MD salts are more stable than the bacitracin base, and presently are the compounds most commonly used in animal feeds. Because feeds vary greatly in composition, bacitracin assay is not without problems. The AOAC method (16) recommends prewashing samples of complete feeds with acetone to remove fat before extraction of bacitracin. In 1969, Craig (17) used petroleum ether for the prewashing step. This implied that fat or other feed ingredients interfered in the analysis of bacitracin, especially in high-energy feeds (5–10% fat) used by broiler producers. However, no precise studies were made to substantiate this effect. Gallagher et al. (18) studied the effects of salts (NaCl and $CaCl_2$), zinc, and complexing agent (EDTA) on the growth of *Micrococcus flavus*. No direct correlation, however, was made on the effect of such ingredients on bacitracin assay in feeds. Grynne et al. documented the only evidence of interference (19), reporting that the high copper content of feeds caused a negative

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bias, and the removal of copper improved recovery.

Generally, the microbiological determination of bacitracin falls into 2 categories: plate or turbidimetric assays. Plate assay is most commonly used. Turbidimetric assay using *Staphylococcus aureus* (ATCC 10537) is described in the *Code of Federal Regulations* (20) for determining pharmaceutical grade bacitracin products. In feeds, *S. faecium* (formerly *S. faecalis*) is the preferred organism for zinc bacitracin analysis. The turbidimetric technique is not widely used among feed analysts. However, as more feed laboratories acquire the necessary equipment, development of turbidimetric methods for analysis of bacitracin in feeds should follow rapidly. Such methods may be preferable over diffusion assays because of higher precision and accuracy, and lower cost.

Scope of Review

All methods used in the analysis of bacitracin in feeds are reviewed. No attempt, however, is made to present the technical details of analytical methods. Appropriate literature references are noted. Results of AOAC collaborative studies in the United States are shown. European literature was reviewed but no collaborative studies similar to those conducted by AOAC in the United States were found. However, reference is made to results of informal collaborative investigations among member countries of the European Economic Community (EEC). Data relevant to accuracy, precision, applicability, sensitivity, and limit of detection are presented.

Sampling

Representative sampling of feeds is important to the accuracy of results. Animal feeds are mixtures of large numbers of components with greatly different particle sizes and densities. Heterogeneity of samples can be a common cause of error. Some workers (21) suggested the use of a large sample weight, e.g., 30 g, for analysis of samples containing 100 g bacitracin/ton, to minimize the inhomogeneity problem. AOAC methods **7.001** and **7.002** (16) discuss sample collection and grinding. Because bacitracin can be readily adsorbed on feed surfaces, which will affect complete extraction, fine grinding of feeds should be avoided; a particle size of 1.0–1.5 mm is appropriate.

Meaning of Potency

When the first assay method for bacitracin was introduced, a strain of Group A hemolytic *Streptococcus* (1) was the test organism. A tentative standard unit was defined as the amount of bacitracin which when diluted about 1:1000 in 2 mL infusion broth will inhibit the test organism. Since then, the definition has been changed; it is now based on the Second International Standard which contains 74 international units (IU)/mg. The International Standard can be obtained from WHO International Laboratory for Biological Standards, National Institute for Medical Research, Mill Hill, London, N.W. 7, UK. The Food and Drug Administration zinc bacitration standard (available from USP Reference Standards, 12601 Twinbrook Pkwy, Rockville, MD 20852) contains 62 IU/mg. The potency of commercial feed grade bacitracin products is variable. The feed industry, however, has accepted the definition of 42 IU/mg. Currently, this is the basis of theoretical potency (label) of the antibiotic in feeds. In the United States, such potency is expressed as g bacitracin activity/lb or ton feed. In other countries, activity is usually expressed as ppm and may be based on the International Standard, i.e., not on 42 IU/mg. In such cases, the basis of activity should be clearly indicated.

METHODS

Microorganisms

Plate assay.—Maintenance of stock cultures and preparation of inocula for *M. flavus* ATCC 10240 and *Sarcina subflava* ATCC 7468 are discussed in **42.199 (a,c)** (16).

Turbidimetric assay.—*S. faecium* ATCC 8043 (formerly *S. faecalis*) is the test organism (22).

Culture Media

(a) *Antibiotic mediums 1 and 2.*—See **42.196 (a,c)** (16).

(b) *Antibiotic medium 3.*—See **42.196 (n)** (16).

Reagents

(a) *Buffer solutions.*—Pyridine-buffer solution (pH 6), 40% pyridine buffer (pH 6.5), 1% phosphate buffer (pH 6), 5% phosphate buffer (pH 6.5), see **42.197 (h, i, f,** and **d,** respectively).

(b) *Acidified methanol.*—2% concentrated HCl in methanol.

(c) *Sodium sulfide solution.*—0.5M (23).

Apparatus

(a) *Heating mat, aluminum air sparger, cylinders, and petri plates.*—See **42.198 (d, e, a, and b,** respectively).

(b) *Flat glass trays.*—20 × 20 cm with lids.

Table 1. Collaborative results for bacitracin activity in broiler ration[a]

Statistic	Sample No.[b] 1	2	3	4	5	6	7	8
Label, g/ton	4.4	4.4	6.6	6.6	22.0	22.0	44.0	44.0
Av. bacitracin found,[c] g/ton	3.5	3.2	5.0	4.9	22.2	22.5	43.1	42.7
Rec., %	80.5	71.8	75.5	74.7	101.0	102.2	97.9	96.9
Coeff. of var., %	29.9	30.1	29.9	26.9	11.7	13.4	11.0	11.8

[a] Ref. 26
[b] Samples 1, 3, 5, and 7 contained zinc bacitracin. Samples 2, 4, 6, and 8 contained MD bacitracin.
[c] Values represent average from 8 laboratories.

(c) *Stainless steel borer.*—11 mm id, beveled and sharpened on the inside edge.

Assay Methods in the United States

With the increased use of antibiotics in feeds, AOAC, in 1953, appointed an Associate Referee on antibiotics in feeds. Shortly thereafter (24), the Referee initiated a study to evaluate methods of analysis for several antibiotics in high potency feed materials (premixes and feed supplements). Among the samples sent to collaborators, one sample contained 6.544 g zinc bacitracin/lb (courtesy of Merck and Co.). Ten laboratories completed the study. The assay method was essentially the same as the current AOAC method **42.202-42.205** used for high potency supplements except that no HCl was used with pyridine buffer for extraction (25). Bacitracin recovery was 99% of theoretical, and the 95% confidence interval was 84.9-113.1. The report was only preliminary, and statistical evaluation appeared incomplete. Good antibiotic recovery obtained in that study was surprising because high acidity (pH <2) is required for complete extraction of zinc bacitracin. However, pharmaceutical grade zinc bacitracin was used for sample fortification.

Since publication of that report in 1957, no further studies were conducted until 1965 when Craig (26) reported on the pyridine method for bacitracin assay in complete feeds. Four samples were prepared by mixing zinc bacitracin with corn-soybean broiler ration at 4.4, 6.6, 22, and 44 g/ton. Another 4 samples of the same feed contained MD bacitracin at the same concentrations. For antibiotic levels of 22 and 44 g/ton, the dose-response line was prepared in 5% pH 6.5 phosphate buffer. For the lower antibiotic levels (4 and 6 g/ton), standard solutions were prepared in phosphate buffer containing material from autoclaved feed. Also, before extraction, interfering substances were removed from samples by acetone washing. Bacitracin was extracted from the feed with HCl and 40% pyridine buffer. Methanol was added to precipitate undesirable proteins if present. Pyridine was evaporated with the air sparger, and after pH adjustment to 6.5, sample extract was diluted to 0.10 unit/mL (reference concentration). The method is the current official first action method, **42.206-42.210.** Results showed recovery of 71.8-80.5% for the 4 and 6 g/ton samples and 96.9-102.2% for the 22 and 44 g/ton samples (Table 1). The coefficients of variation averaged about 30% for the lower potency levels compared with about 12% for high levels. The method appeared to be applicable to both types of bacitracin, although no statistical evidence was presented. Furthermore, only one type of feed was evaluated.

In 1969, the method was modified for low level (4-10 g/ton) assays (17). Neomycin (1.0 μg base/mL) was added to the medium to increase zone size (0.04 unit/mL as reference concentration). Sample cleanup with acetone was carried out with the Goldfisch fat extraction apparatus. The method remained essentially the same as in the earlier study. Results showed no loss of potency in supplements containing feed-grade zinc bacitracin, manganese bacitracin, and natural bacitracin. In the case of MD bacitracin, however, there was 25% loss of potency. When petroleum ether was substituted in the cleanup step for acetone, all 4 types of bacitracin retained their original activity. Comparison was also made in this study of standard dose-response lines prepared in plain pH 6.5 buffer and in blank feed extracts. Nonzinc pharmaceutical grade bacitracin was added to feed extracts as follows: broiler ration at 4 and 6 g/ton; 38% poultry concentrate at 4 g/ton; sheep ration at 7 g/ton; and sow-15 ration at 10 g/ton. Further dilutions to correspond to different concentrations on the standard response line were made

Table 2. Difference between dose–response lines in feed extract and in pH 6.5 buffer [a]

Type of feed (label)	% difference in activity at (units/mL)		
	0.02	0.04	0.08
Broiler ration (4 g/ton)	1.0	5.0	8.1
Broiler ration (6 g/ton)	0	0.8	3.7
38% poultry concentrate (4 g/ton)	12.5	7.5	0.1
Sheep ration (7 g/ton)	4.0	0	2.5
Sow-15 ration (10 g/ton)	2.6	5.3	7.3
Buffer vs buffer (control)	2.5	2.5	2.5

[a] Ref. 17.

with blank feed extracts. Results showed the curves prepared in buffer and in blank feed extracts of the 4 types of feed to be similar (Table 2). When feed-grade zinc bacitracin was mixed with the broiler ration at 4 g and 6 g/ton, the average of 4 assays at 4 g/ton assayed against standard in buffer was 2.5% higher (Table 3) than that for the same aliquots assayed against a standard curve in blank feed extract. Similarly, the broiler ration containing 6 g/ton was 8.5% higher vs the buffer standard compared with the blank feed standard. Because the coefficients of variation ranged between 2.3 and 11.6% and no tests for significance were made in the study, not much emphasis can be attached to this positive bias in results.

In 1976, Fassbender and Katz (27) modified the double dose technique of Knudsen and Randall (28). They suggested that on each plate, 2 opposing cylinders be filled with the lowest concentration of standard, 2 with the highest concentration, and 2 with the unknown. The lowest standard potency was 0.1 unit/mL and the highest 0.4 unit/mL. This 4-fold range of concentrations was preferred over the customary 16-fold range because of linearity of the relationship between the diameter of the zone of inhibition and logarithm of concentration in the shorter range. These investigators found that when premixes were extracted with acidic 50% methanol and diluted with pH 6 buffer, the 2-point plating system was as valid as the AOAC design. Methanol extraction was comparable to extraction by the AOAC pyridine method; F values showed no statistical difference ($P < 0.05$) (Table 4). For complete feeds (10–100 g/ton), the samples were prewashed with acetone, allowed to dry, acidified with 0.36N HCl, then neutralized with 0.36N NaOH. The antibiotic was extracted with 50% aqueous dimethylformamide (DMF) at 45°C. After cooling, the samples were diluted with triple strength pH 6 buffer. The authors claimed the method to be comparable to the AOAC pyridine method (Table 5).

Preliminary experiments in our laboratory did not confirm these findings. The AOAC pyridine extraction method appeared superior to DMF extraction. This might have been due to the poor quality of zones of inhibition of *M. flavus* on antibiotic medium 2 used by Fassbender and Katz (27) compared with antibiotic medium 1 used by the AOAC method. The modified 2-point assay and the narrower range of standard concentrations appeared to be a better assay design than the AOAC 5-point design.

Another study was conducted in 1976 (22) to

Table 3. Comparison of results for broiler ration using standards in buffer and in blank feed extract [a]

Formulated potency	Bacitracin found			
	4 g/ton		6 g/ton	
	Std in buffer	Std in feed blank	Std in buffer	Std in feed blank
Av., g/ton [b]	3.9	3.8	6.1	5.6
Rec., %	97.5	95.0	101.6	93.3
Coeff. of var., %	11.6	10.2	9.7	2.3

[a] Ref. 17.
[b] Values represent average of 4 aliquots.

Table 4. Comparison of methanol and AOAC pyridine methods for determination of bacitracin in premixes [a]

Methods [b]	Premix 1 [c]		Premix 2 [c]	
	Methanol extraction	AOAC pyridine extraction	Methanol extraction	AOAC pyridine extraction
Av., g/lb [d]	27.3	29.3	38.9	37.4
Rel. std dev.	10.8	10.4	10.5	12.2

[a] Ref. 27.
[b] Two-point assay design was used.
[c] Premix 1 contained 25 g MD bacitracin/lb, and Premix 2 contained 40 g zinc bacitracin/lb.
[d] Average of 10 replications.

evaluate methanol and pyridine extraction of bacitracin in feeds by plate and turbidimetric methods. The photometric technique involved use of *S. faecium* (ATCC 8043) as test organism and Penassay broth as final assay medium. The broth contained 10^{-4}M $ZnCl_2$ to increase assay sensitivity, 0.3 mL polysorbate 80/100 mL medium to minimize interference by fat, and 1 mL 50% glucose solution/100 mL medium to enhance growth of test organism. Complete feed samples were prewashed with petroleum ether, then extracted with pyridine or methanol. The pyridine method was essentially the same as recommended by Craig (17). The evaporation step was necessary because pyridine considerably inhibited *S. faecium* (turbidimetric method) and *M. flavus* (plate method). For methanol extraction, the samples were treated with 1N HCl (pH of extracts <2.0) for 5 min followed by addition of 5% methanol–phosphate buffer (1%, pH 6.5). After centrifugation and filtration through glass wool, pH was adjusted to 6.5, and the samples were diluted with methanol buffer. The standard dose-response line was prepared in the same diluent. The concentration of methanol in this diluent should not exceed 10%, to avoid any interference with growth of test organism for turbidimetric and diffusion methods.

When zinc bacitracin standard solutions were added to swine and broiler rations and the antibiotic was extracted by pyridine, recovery by the turbidimetric method (93.1–102.6%) agreed well with the plate method (88–112%). Relative standard deviation values (about 10%) indicated the same precision for both methods. Methanol extraction yielded poor recovery (60–70%) by the turbidimetric method. Before this assay can be successfully developed, it is necessary to investigate the effect of substances that are extracted from feeds by methanol on the photometric response of *S. faecium*. For the plate assay, recovery (95.5–118.6%) and precision (rel. std dev. 5.26–9.22%) by methanol extraction were good for both feeds.

When feeds were fortified (50–200 g/ton) with commercial zinc bacitracin premix and studied collaboratively by 3 laboratories, recovery by plate assay for pyridine extraction was about 73% for the swine ration and 94% for the broiler ration. For methanol extraction, there was some

Table 5. Comparison of DMF and AOAC pyridine methods for determination of bacitracin in complete feeds [a]

Statistic	Level of fortification, g/ton															
	Feed A [b]								Feed B [b]							
	100		50		25		10		100		50		25		10	
	DMF [c]	AOAC	DMF	AOAC	DMF	AOAC	DMF	AOAC	DMF	AOAC	DMF	AOAC	DMF	AOAC	DMF	AOAC
Av., g/lb [d]	79.3	84.3	46.2	46.8	25.4	26.3	13.0	8.2	76.7	101.9	42.4	61.2	30.3	35.1	13.8	6.9
Rel. std dev.	14.9	12.6	8.0	12.6	15.7	19.0	22.3	19.5	8.6	17.7	7.3	9.3	13.2	18.3	16.7	43.5
Ratio AOAC/DMF	1.06		1.01		1.04		0.63		1.32		1.44		1.16		0.50	

[a] Ref. 27
[b] Feed A represented starting mash (Rutgers ration 68-S), and feed B was 16% laying mash.
[c] For DMF method, feeds were extracted with 50% dimethylformamide; for AOAC method, feeds were extracted with 40% pyridine buffer as in **42.208** (16).
[d] Average of 9 replications.

positive bias (about 108%) for both feeds. This bias did not appear to be due to the presence of methanol because phosphate buffer solutions containing up to 20% methanol showed no inhibition of test organism.

For feeds fortified with MD bacitracin premix at levels of 20, 50, and 100 g/ton, 4 laboratories found antibiotic recovery to be higher (119–132%) by the plate methanol method than by the pyridine method (97–104%). That study (22) indicated that the turbidimetric method was applicable only for the analysis of zinc bacitracin by pyridine extraction. The plate method showed some positive bias by methanol extraction for either zinc or MD bacitracin.

That study (22) recommended that all collaborating laboratories use the same zinc bacitracin standard; therefore another study (29) was conducted to evaluate the pyridine method for the 2 types of bacitracin salts in 2 types of complete feeds at 2 levels of fortification. In an attempt to unify the basic method for premixes and complete feeds, the study included 2 high-potency samples. The current AOAC method (**42.202–42.205**) specifies a 1% pH 6 phosphate buffer and different concentrations for the standard dose-response line for premixes, and a 5% pH 6.5 phosphate buffer for complete feeds. Each laboratory received a culture of the test organism *M. flavus* (ATCC 10240), 8 complete feeds (4 poultry and 4 swine), and 2 premixes (Premix I and II with theoretical label of 50 g MD bacitracin/lb and 40 g zinc bacitracin/lb, respectively). These premixes were used to fortify the complete feeds. Two samples of each type of feed were fortified with zinc bacitracin or MD bacitracin each at about 10 g/ton. Similarly the other 2 samples each were fortified at about 100 g/ton (one poultry sample was fortified with 150 g zinc bacitracin/ton although label remained 100 g/ton). All collaborators received zinc bacitracin standard (of the same lot) with potency label of 61.8 units/mg. The collaborators were instructed to follow the pvridine extraction method using neomycin-sensitized agar (one layer of medium) and petroleum ether cleanup. Samples were diluted according to specified scheme (2 dilutions prepared), and the entire assay procedure was repeated on a second day.

The comments made by collaborators (29) gave some insight into the nature of problems encountered with the method. For example, some collaborators did not report results because of very large and poorly defined zones of inhibition which could not be accurately measured. Other collaborators experienced difficulty in determining when pyridine was completely evaporated. Results for these laboratories showed positive bias apparently due to incomplete evaporation of pyridine. Addition of a small amount of methanol before the end of evaporation and increasing the drying time under the air sparger reportedly improved the results. Another group of collaborators allowed the temperature to increase too high during evaporation, e.g., one collaborator did not use an air sparger but dried samples in small dishes under hot blowing air. Results by these laboratories showed negative bias, indicating destruction of bacitracin activity. There were other modifications in the method which apparently had no effect on results. For example, one laboratory, after evaporation, suspended the dried materials in pH 6.5 buffer and refrigerated the plates until the following day. Another collaborator used rotary evaporation for drying. Additional data were also presented by one laboratory which showed no significant difference between evaporation and no evaporation for premix samples. Finally, most collaborators preferred an extracting agent other than pyridine.

Results in that study (29) showed no significant difference between dilutions within a day or between days for each sample. The type of bacitracin or type of feed did not significantly affect results. There was some difference in results between MD and zinc bacitracin in premixes which approached significance (Table 6). Also, the overall recovery of MD bacitracin was 91% compared to 101.5% for zinc bacitracin. This may have been due to less solubility or some destruction of MD bacitracin by the acidity of the extracting agent. Future studies should investigate this problem. Statistical evaluation showed large coefficients of variation (about 13% for premixes and 15–30% for complete feeds). This is indicative of serious operational difficulties, which was supported by the findings that only 9 of 15 laboratories were able to differentiate between the label (100 g/ton) and the theoretical fortification level (150 g/ton) of the poultry feed sample. The coefficient of variation for this sample was the highest (about 27%) among the complete feeds. When results submitted by the laboratories were ranked (30), 3 laboratories were outside the score limit and 2 others approached the upper limit. Thus, about one-third of the laboratories showed either very high or very low results. The apparent source of error was the pyridine evaporation step. Some laboratories were unable to completely eliminate pyridine (positive bias), whereas others

Table 6. Collaborative study of bacitracin activity in 2 premixes and 2 complete feeds by the pyridine method [a]

	Premix I [b]	Premix II	Sample A	B	C	D	E	F	G	H
Theoretical label [c]	50	40	10	10	10	10	100	150	100	100
Type of bacitracin [d] used	MD	Zn	MD	Zn	MD	Zn	MD	Zn	MD	Zn
Av. bacitracin found, [c]	45.5	40.6	9.3	10.6	9.8	10.8	99.0	146.3	90.7	99.0
Coeff. of var., %	12.9	13.6	23.1	17.8	21.0	13.7	19.0	26.8	16.4	15.3
No. of collab. labs.	17	17	14	13	15	14	17	17	16	16

[a] Ref. 29.

[b] Premixes I and II represent MD bacitracin and zinc bacitracin with theoretical activities of 50 and 40 g/lb, respectively. Samples A, B, E, and F represent poultry feed; samples C, D, G, and H, swine feed.

[c] Bacitracin potency expressed as g/lb for premixes and g/ton for complete feeds.

[d] MD = bacitracin methylene disalicylate; Zn = zinc bacitracin.

probably exposed sample extracts to higher temperatures causing destruction of the antibiotic (negative bias). The Associate Referee recommended that the pyridine method be discontinued.

In the earlier study of the methanol method (22), 1% pH 6.5 phosphate buffer containing 5% methanol was used as diluent for standard and samples. Further experiments showed that bacitracin activity is influenced by pH, concentration of phosphate buffer, and concentration of methanol. Table 7 shows that bacitracin activity was somewhat depressed in 5% methanol especially in 1% phosphate buffer (solution 5) compared to methanol in 5% phosphate buffer (solution 2). Both buffers (without the antibiotic) showed no inhibition of test organism. At 15% methanol concentration (solution 3) there was positive bias (recovery of 121.1%). Similarly the effect of pH was significant. Solution 6 containing 0.04 unit bacitracin/mL, when diluted with pH 6 buffer, gave 0.0472 unit/mL, indicating that 5% phosphate buffer containing 5% methanol is a better diluent than methanol–1% buffer.

Accordingly, the methanol extraction method was re-evaluated by 5 laboratories. Each collaborator was provided with 2 premix samples (Premix I with label of 50 g MD bacitracin/lb and Premix II labeled to contain 40 g zinc bacitracin/lb) and 4 complete feed samples (2 poultry and 2 swine). Each poultry or swine feed sample was fortified with each bacitracin salt at 100 g/ton using each respective premix. The collaborators were also provided with the same lot of zinc bacitracin standard, a culture of *M. flavus*, and the dilution scheme for each sample. The methanol method was the same as that used in an earlier study (22) except that the diluent for standard and sample was 5% methanol–pH 6.5 phosphate buffer (5%).

Statistical evaluation of assay results was made by applying the Dixon test (30) for outliers to the 2 sets of values (2 dilutions) for each day. The

Table 7. Effect of concentration of phosphate buffer, pH, and methanol on bacitracin activity [a]

Parameter	Solution [b] Std [c]	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Buffer pH	6.5	6.5	6.5	6.5	6.5	6.5	6.0
Phosphate buffer, % concn	5	5	5	5	1	1	5
Methanol concn, %	0	0	5	15	0	5	0
Bacitracin activity found, unit/mL	0.040	0.0395	0.0366	0.0485	0.0350	0.0290	0.0472
Recovery, %	—	98.7	91.5	121.1	87.8	72.5	118.1
Coeff. of var., %	—	6.0	3.1	3.9	4.5	7.7	5.8
Number of detns	—	15	12	7	17	14	8

[a] Ref. 31.

[b] Each solution contained theoretically 0.04 bacitracin activity unit/mL. Diluents for solutions were: No. 1 (5% pH 6.5 buffer); No. 2 (5% pH 6.5 buffer containing 5% methanol); No. 3 (5% pH 6.5 buffer containing 15% methanol); No. 4 (1% pH 6.5 buffer); No. 5 (1% pH 6.5 buffer containing 5% methanol); and No. 6 (5% pH 6 buffer).

[c] Activity was determined on neomycin-sensitized plates vs standard solutions prepared in 5% pH 6.5 buffer.

Table 8. Average bacitracin found [a] in premix and complete feeds by methanol method [b]

	Theoretical potency					
			Poultry feed		Swine feed	
Coll.	Premix I, MD 50 g/lb	Premix II, Zn 40 g/lb	MD 100 g/ton	Zn 100 g/ton	MD 100 g/ton	Zn 100 g/ton
1	—[c]	46.2	88.6	106.6	94.3	95.1
2	41.6	42.3	96.0	114.2	98.7	111.1
3	42.7	45.0	73.9	101.7	82.7	86.6
4	42.3	40.0	103.5	106.2	80.8	91.7
5	40.4	42.3	92.7	95.4	94.2	103.3
Overall av.	41.8	43.2	90.9	104.8	90.2	97.6
Coeff. of var., %	2.41	5.68	12.09	6.60	8.75	9.94
Rec., %	83.6	105.8	90.9	104.8	90.2	97.6

[a] Average of day 1 and day 2.
[b] Ref. 31.
[c] Value reported for dilution 1 on day 2 was an outlier. Average of dilutions on day 2 was also an outlier. Thus, average of day 1 and day 2 was deleted for Collaborator 1.

Student *t*-test (32) was used to ascertain if differences were significant. The 2 dilutions were averaged (even though one result may have been previously rejected) to give one set of values for each day. This was because the average of 2 dilutions may still be statistically valid. The between-days variability was similarly evaluated. Results showed overall recovery of MD bacitracin (83.6%) to be lower than that of zinc bacitracin (105.8%). Similarly, poultry and swine rations fortified with these premixes showed about 90% recovery for MD bacitracin and 101% for zinc bacitracin (Table 8). There was no significant difference between days or due to the type of feed or kind of bacitracin. The collaborators found the methanol method to be simpler and more precise than the pyridine extraction method. Further experiments are needed to investigate the reasons for lower recovery for MD bacitracin. According to these results, the Associate and General Referees on bacitracin in feeds have recommended that AOAC adopt this method as official first action for zinc bacitracin analysis (31).

Assay Methods in Europe

In 1971, Grynne, in Norway, reported on a methanol method (15) for zinc bacitracin assay. Feeds containing 20–350 ppm (18–318 g/ton) were extracted 2 min with acid methanol (pH of extract about 2), then shaken 20 min with 5% phosphate buffer (pH 6.5). After centrifugation, the extract was diluted with buffer to contain 0.05–0.1 unit/mL and assayed against zinc bacitracin in buffer. For lower potency feeds (5–20 ppm, or about 4.5–18 g/ton), the samples were prewashed with acetone and allowed to dry completely. Extraction of the antibiotic was the same as for the higher potency feeds except that after centrifugation, a 20 mL aliquot was adjusted to pH 6.5 and evaporated to dryness at 30°C in a rotary evaporator. The residue was redissolved and diluted in phosphate buffer to 0.05–0.10 unit/mL. Standard solutions were prepared in unsupplemented blank feeds. When not available, the standard addition technique was followed, i.e., adding a known increment of the antibiotic to a second weighing of the sample, determining recovery of added increment, then correcting the potency of the sample.

In higher potency feed, there was no need for solvent evaporation because it was easy to keep methanol concentration below 15%. At this concentration, the apparent change of antibiotic potency (0.1 unit/mL) was −5%. For lower potency feeds, however, this was not possible necessitating the rotary evaporation step. The findings by Grynne (15) were based on comparison between the sizes of zones of inhibition in presence of solvent and in its absence. The zones of inhibition were reported to 0.02 mm. For example, in absence of methanol, 0.10 unit/mL zinc bacitracin gave an average zone of inhibition of 17.10 mm. In the presence of 10 or 15% methanol, the average zone diameter was 16.94 mm. Hence the apparent change of potency was reported to be −5%. In order to evaluate these conclusions it is important to remember that accuracy of zone measurement could be a factor limiting precision of diffusion assays (33, 34). For example, an error of 0.1 mm in measuring zone diameter in AOAC chlortet-

Table 9. Comparison of methanol and pyridine extraction for determination of bacitracin in feeds [a]

Feed sample	Bacitracin found, ppm		
	Methanol extraction		
	Std in buffer	Std in blank feed	Pyridine extraction
	Procedure A [b]		
1 [c]	19	22	21
3	27	31	22
6	48	42	44
9	105	113	95
12	327	347	333
	Procedure B [b]		
17	3.6	3.5	3.9
22	10.0	10.0	9.8
27	21.0	20.0	18.0

[a] Ref. 15.
[b] Procedure A required no evaporation of methanol, whereas in procedure B methanol was evaporated.
[c] Each result represents the average of 3 separate determinations.

racycline assay, caused 2.5% error in potency (35). In general, Grynne (15) showed the methanol to be comparable to the pyridine method (36) for determining zinc bacitracin in feeds (Table 9).

There was some pertinent information missing from this published report (15), e.g., identity of test organisms, general composition of feeds used in the study, and accuracy and precision of results when the standard addition technique was followed. Furthermore, in some experiments, cylinders were used for assay, whereas in others, holes were cut in agar media. This method, however, was the first published report in which an organic solvent less toxic than pyridine was used for bacitracin assay.

In 1973, the method was modified (19) to minimize interference by copper when present in high concentrations especially in the assay of low potency feeds. This was accomplished by adding 0.4 mL ammonium sulfide solution (later changed to sodium sulfide) following the addition of the acidified methanol for extraction. The evaporation step eliminated methanol as well as interference by sulfide with the test organism. Bacitracin recovery was greatly improved by precipitation of copper sulfide. For example, at 6.0 ppm (5.4 g/ton) zinc bacitracin and 188 ppm copper, only 1.7 ppm bacitracin was found by the regular method and about 6 ppm by the modified method. This was a good demonstration of the effect of metal interference in bacitracin assay (Table 10).

Table 10. Effect of copper on determination of bacitracin in feeds [a]

Theoretical content, ppm		Bacitracin found, [b] ppm	
Bacitracin	Copper	Method I [c]	Method II
6.0	188	1.7	6.0
10.0	190	6.6	10.3
20.0	200	15.7	21.3
20.0	300	9.9	18.9

[a] Ref. 19.
[b] Bacitracin was extracted by methanol and each result represents the average of 3 determinations.
[c] For method I, interference by copper was not eliminated; for method II, copper interference was minimized by sulfide precipitation.

These assay methods and other studies conducted among the countries of the EEC were discussed in a report by the Sub-Committee on Antibiotics in Animal Feeding Stuffs (23).

The general consensus was that preparation of dose-response line in unmedicated feeds is of no importance because such samples are seldom available. Experiments to destroy zinc bacitracin in feed extracts by heat proved impractical because of resistance of bacitracin solutions to destruction by heat (complete destruction of antibiotic activity requires 45 min at 15 psi). Furthermore, this drastic treatment sometimes resulted in unwanted effects on other feed ingredients. Several experiments were conducted to study the effect of different extraction conditions. Use of hexametaphosphate and disodium ethylenediaminetetraacetate as sequestering agents to prevent formation of precipitates which

might lead to loss by coprecipitation of bacitracin had no effect on bacitracin recovery. Stricter control of pH of buffer and of sample extracts resulted in slight improvement in recovery. Acetone prewash was advantageous in removing fat and other interfering substances. Feed samples containing 5–20 ppm zinc bacitracin gave only 60–70% recovery by the standard methanol method. Recoveries were approximately 100% when corrected by the standard addition technique, which indicated that zinc bacitracin was either not being completely extracted or there was interference by some feed ingredients.

Other experiments were performed to eliminate some assay problems. When phosphate buffer was substituted by 2-amino-2-(hydroxymethyl)propane-1,3 diol (tris) buffer and methanol, 2 laboratories assaying 0.100, 0.050, and 0.025 unit of bacitracin/mL recovered over 90% of the activity. However, the tris–methanol buffer (with no bacitracin) gave appreciable zones. Also, it was difficult to redissolve the solids after evaporation. Heavy contamination was noted on the plates that were incubated at 37°C, and sensitivity was not high. When 2,2′-dipyridyl was used to eliminate possible interference from iron, there was no special advantage.

For low level feeds (as low as 10 ppm) extracted with methanol, partial evaporation of methanol and pH adjustment to 6.5 resulted in some positive bias in recovery (100–120%). Further investigations showed this to be due to some inhibition by blank feeds. Accordingly, the rotary evaporation step was re-introduced.

The EEC report also evaluated several techniques used in microbiological assay. Wells cut in agar were superior to cylinders or paper discs in formation of uniform zones of inhibition. The cutting of wells by cork borer was considered poor. Instead of using brass tubing with a bevel on the outside of the tube, it was essential to use a thin walled steel tube with sharp bevel on the inside. To minimize contamination by copper, the tube should be made of ferrous metal rather than brass. A 2 × 2 dose Latin square was superior to the random 4 × 4 design, because the response tended to become nonlinear over the extended range required for the 4 × 4 design. This difficulty was resolved by adding neomycin to the medium. Other advantages of neomycin included increased assay sensitivity and control of contamination during incubation at 37°C.

The final method suggested in the report was essentially the same methanol method reported by Grynne (15) except that sulfide salt was added in the presence of high copper content. The Latin square 2 × 2 design was followed and the volume of solution (0.2 mL) necessary to fill each hole should be predetermined and measured accurately.

Conclusions

Plate diffusion assay is the method of choice for analysis of zinc or MD bacitracins. For feed products containing >20 g bacitracin/ton and for higher potency premixes (bacitracin in g/lb), methanol extraction is superior to pyridine. Statistical evaluation of AOAC collaborative studies of premixes showed no component of variance (coeff. of var., 2–6%) for the methanol method and large coefficient of variation (about 13%) for the AOAC pyridine method. Comparable values for complete feeds were 6–12% and 15–30%, respectively. For potency level <11 ppm (about 10 g bacitracin/ton), no AOAC collaborative studies were done. Future investigations at low antibiotic levels are necessary to evaluate the effect of feed matrix interference on antibiotic recovery, precision, accuracy, and sensitivity of the analytical method.

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INDUSTRIAL CHEMICALS

Gas Chromatographic Determination of Trialkyl/Aryl Phosphates in Drinking Water, Following Isolation Using Macroreticular Resin

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An XAD-2 screening method developed previously for organophosphorus pesticides has been extended to determine trialkyl/aryl phosphates in drinking water at the ng/L level. Recovery studies at 1, 10, and 100 ng/L levels were carried out by fortification onto XAD-2 resin and by direct on-stream fortification of drinking water. Recoveries were >70% for tri-n-butyl, tris(2-chloroethyl), tri(2-ethylhexyl), tributoxyethyl, triphenyl, and tricresyl phosphate. Triethyl phosphate recoveries were about 25%. Analysis of drinking water samples collected at 6 eastern Ontario water treatment plants revealed the presence of several trialkyl/aryl phosphates at levels ranging from 0.2 to 75 mg/L.

Trialkyl/aryl phosphate esters are widely used as flame retardants in plastics and as fire-retardant hydraulic fluids; smaller amounts are used in lubricants, air filter media, adhesives, and coatings (1, 2). Although significant quantities may have been released inadvertently to the environment (1), there are few reports of the presence of trialkyl/aryl phosphate esters in the environment (1, 3–9).

Murray (8) extracted phosphate esters from water with chloroform and, following hydrolysis of the phosphates and silylation, determined the silylated phenols by gas chromatography. Sheldon and Hites (6), Saegar et al. (9), and Clarke (7) used methylene chloride to extract the phosphates from water and analyzed the extracts by gas chromatography using flame photometric (7), flame ionization (9), and mass spectrometric (6) detection. However, detection limits of these methods were greater than 10 μg/L (7); trialkyl/aryl phosphates should be present in drinking water at much lower concentrations, so a more sensitive method has been developed.

LeBel et al. (10) reported a method for the isolation and concentration of organophosphorus pesticides from drinking water at the ng/L level, using Amberlite XAD-2 macroreticular resin. This method has been extended to determine trialkyl/aryl phosphates in drinking water at ng/L concentration; samples from 6 eastern Ontario treatment plants were used.

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METHOD

Reagents

Use solvents and chemicals as previously described (9) except for:

(a) *Purified water.*—Pass water from Millipore Super-Q system through Amberlite XAD-2 resin cartridge at ca 140 mL/min and store in clean, capped amber bottles with Teflon-lined caps.

(b) *Glass wool.*—Wash with acetone, methylene chloride, and hexane. Store in clean bottles with Teflon-lined caps.

(c) *Standard solutions.*—All phosphates were obtained from Chem Service Inc. (West Chester, PA 19380) except tri-*o*-, tri-*m*-, and tri-*p*-tolyl phosphate (Eastman Kodak, Rochester, NY 14650), tris(1,3-dichloropropyl)phosphate (RFR Corp., Hope, RI 02831), and tri(2,4-xylenyl)-phosphate (Chrompack Canada, Blenheim, Ontario, Canada NOP 1A0). Prepare 500 μg/mL stock solutions in acetone and working standard solutions in acetone as required.

Apparatus

(a) *Ultrasonic bath.*—Bransonic, Model 220.

(b) *Centrifuge.*—Table top, International Clinical Centrifuge. Operate at 1600 rpm.

(c) *LC pump.*—Waters Associates (Milford, MA 01757), Model 6000.

(d) *Gas chromatograph.*—Perkin-Elmer Model 910 equipped with nitrogen-phosphorus selective detector (NPD). Column parameters and operating conditions: 1 m × 2 mm id glass column packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q, initial temperature 160°C for 2 min, program 6°/min to 235°C, hold 10 min, hold post-program temperature 6 min at 240°C; or, 1.8 m × 2 mm id glass column packed with 3% OV-101 on 80–100 mesh Chromosorb 750, initial temperature 150°C for 2 min, program 8°/min to 230°C, hold 10 min, hold post-program temperature 9 min at 235°C, helium carrier gas at 30

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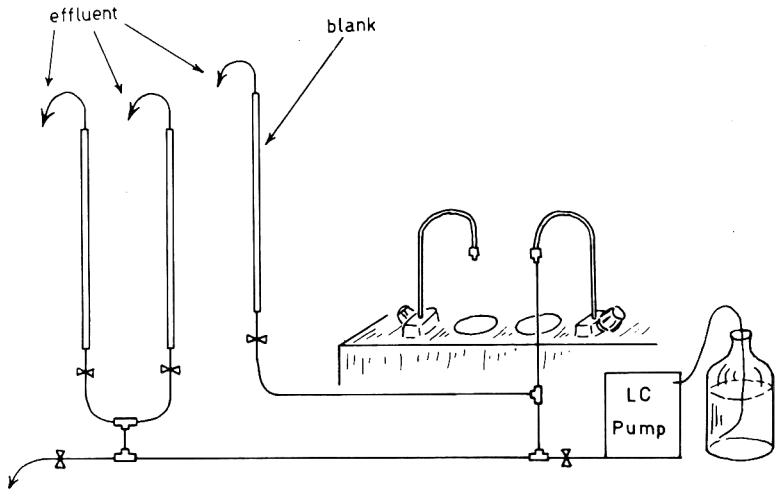

Figure 1. Schematic of continuous fortification using LC pump.

mL/min; or, 25 m × 0.2 mm id methyl silicone (SP 2100) fused silica capillary column (Hewlett-Packard), initial temperature 170°C for 2 min, program 5°/min to 245°C, hold 10 min, hold post-program temperature 5 min at 250°C, helium carrier gas 40 cm/s linear velocity at 28 psig inlet pressure with make-up gas at 25 mL/min. Injector, 250°C; detector, 270°C. Using syringe with special 3 in. needle, introduce aliquot through splitless injector system (SGE) at 70°C, open vent at 20 s, and increase initial temperature to 170°C at 30 s.

(e) *Gas chromatograph–mass spectrometer (GC/MS).*—Finnigan Model 4000, coupled with series 6110 data system and containing a 1.8 m × 2 mm id glass column packed with 3% OV-101 on 80–100 mesh Chromosorb 750. Operating conditions: initial temperature 175°C for 1 min, program 6°/min to 250°C; separator 245°C; ion source 250°C.

Water Extraction

Pass 200 L tap water through Amberlite XAD-2 resin cartridge at 140 mL/min. Elute XAD resin as previously described (10).

Extract 2 L water sequentially with 100, 50, and 50 mL portions of methylene chloride. Dry methylene chloride extracts by percolating through sodium sulfate drying tube, and combine in 500 mL round-bottom flask. Rinse sodium sulfate with 25 mL methylene chloride and add rinse to combined extracts. Concentrate filtrate to near dryness, add ca 10 mL acetone, and reconcentrate to near dryness. (Note: Halogenated solvents adversely affect NPD (11).) Dilute to suitable volume for GC analysis, using acetone as solvent.

Recovery Studies

Direct on-column fortification.—Add aliquot of phosphates spiking solution in acetone, corresponding to 1, 10, or 100 ng/L for equivalent 200-L water sample, to head of cartridge and flush acetone with 500 mL purified water. Invert column, attach to tap water line in series with 1 (or 2) screening XAD-2 cartridge(s) and proceed as previously described for water extraction.

Continuous fortification.—Fortify 4 L purified water with 0.55, 5.5, or 55.0 μg phosphates by adding appropriate amount of stock solution (1 or 10 μg/mL) of phosphates in acetone. Using high pressure LC pump and tee-junction (Figure 1), pump this solution into inlet water line of XAD cartridge at a rate (ca 2 mL/min) calculated to give a fortification level of 1, 10, or 100 ng/L. Pass 200 L fortified water through XAD cartridge, and elute cartridge as previously described.

Extraction from Selected Rubber Seals and O-Rings

With clean scalpel, cut very fine pieces of rubber and accurately weigh ca 25 mg. With mortar and pestle, grind to fine powder with ca 1 g granular sodium sulfate. Transfer to 50 mL round-bottom centrifuge tube equipped with Teflon-lined screw cap. Add 25 mL acetone and ultrasonicate 15 min. Centrifuge 15 min and transfer clear supernate with clean Pasteur pipet to 250 mL round-bottom flask through solvent-

Table 1. Recoveries of trialkyl/aryl phosphates by direct fortification

Phosphate	% Recovery ± SD		
	1 ng/L[a]	10 ng/L[a]	100 ng/L[b]
Triethyl	—	19.9 ± 1.3	28.9 ± 4.9 (10.7 ± 0.1)[a,c]
Tributyl	88.2 ± 4.1	101.2 ± 2.1	97.5 ±2.3
Tris(2-chloroethyl)	82.9 ± 0.5	95.5 ± 1.0	90.3 ± 5.4 (2.4 ± 0.4)[a,c]
Tri(2-ethylhexyl)	62.9 ± 6.9	61.7 ± 6.9	(78.8 ± 8.5)
Tributoxyethyl	125.6 ± 13.9	141.5 ± 9.3	104.5 ± 6.5
Triphenyl	100.5 ± 2.1	107.4 ± 4.5	95.0 ± 3.6
Tricresyl	98.1 ± 1.4	97.1 ± 3.5	95.0 ± 4.8

[a] Two determinations.
[b] Four determinations.
[c] Values in parentheses are for recoveries from a second XAD cartridge when used in series with the first cartridge.

washed sodium sulfate drying tube. Repeat twice and rinse drying tube with 15 mL acetone. Concentrate filtrate to small volume on rotary evaporator, transfer to graduated centrifuge tube, and dilute to required volume in acetone for GC analysis.

Gas Chromatography

Inject aliquot of extract into gas chromatograph with OV-17 column and quantitatively determine amount of unknown or spiked material by comparing its peak area with area of corresponding standard injected under similar conditions. Inject onto OV-101 column for confirmation.

Confirmation by Gas Chromatography/Mass Spectrometry

Inject suitable aliquot of extract into GC/MS apparatus. For qualitative confirmation, compare EI mass spectrum of peak of interest with EI mass spectrum of corresponding standard. For quantitation, compare area of peak of reconstructed mass chromatogram (RGC) with area of corresponding standard RGC peak. For trace levels, use GC-selected ion monitoring MS and compare peak areas for 2 characteristic ions (RGC) with corresponding areas from standards.

Results and Discussion

The method of LeBel et al. (10) developed previously for the determination of organophosphorus pesticides has been extended to include simultaneous determination of trialkyl/aryl phosphates. Recovery studies were carried out by 2 different methods to measure the ability of the resin to concentrate the trialkyl/aryl phosphates from drinking water samples. The first fortification method has been described (10). In the present method, an acetone solution of the phosphates is spiked directly onto an XAD-2 cartridge at a concentration equivalent to 1, 10, or 100 ng/L for a 200-L water sample, and 200 L tap water screened through XAD-2 resin is allowed to pass through the spiked cartridge at 140 mL/min. A blank cartridge is run in parallel for all recovery runs.

Results for recoveries by using the direct fortification method are shown in Table 1. The resin produced good overall recovery with a few exceptions. The low recovery for triethyl phosphate can be explained by its relatively high solubility in water. This results in poor adsorption by the XAD-resin as shown (Table 1, parentheses) by connecting a second XAD-2 cartridge in series with the first cartridge. In addition to loss due to this breakthrough, triethyl phosphate was also found in the aqueous phase usually discarded following elution of the resin. Analysis of this aqueous phase (methylene chloride extraction) accounted for 30% of the triethyl phosphate. Of the phosphates used for fortification, only tris(2-chloroethyl)phosphate was also found in the aqueous phase at 10% of the spiked level.

The apparent high recovery for tributoxyethyl phosphate is explained by the presence of this compound in the XAD-screened tap water used in the fortification studies. This is due to the relatively high level (about 55 ng/L) of tributoxyethyl phosphate in the tap water and about 8% breakthrough of this compound through the XAD-2 cartridge.

It has been questioned whether direct fortification of the XAD resin is a true indication of the

Table 2. Recoveries of trialkyl/aryl phosphates by continuous fortification using an LC pump

Phosphate	% Recovery ± SD		
	1 ng/L[a]	10 ng/L[a]	100 ng/L[b]
Triethyl	—	29.4 ± 2.3	20.7 ± 2.3
		(16.1 ± 0.5)[c]	(14.1 ± 0.4)
Tributyl	78.9 ± 6.5	71.3 ± 7.1	76.6 ± 4.9
	(26.7 ± 0.3)	(15.7 ± 2.9)	(15.1 ± 3.5)
Tri(2-chloroethyl)	81.1 ± 5.6	86.1 ± 3.4	81.1 ± 9.4
	(21.5 ± 0.2)	(19.4 ± 2.9)	(16.5 ± 1.5)
Tri(2-ethylhexyl)	10.1 ± 0.1	9.3 ± 0.4	13.2 ± 3.7
	(3.1 ± 0.1)	(4.1 ± 0.6)	(6.4 ± 2.0)
Tributoxyethyl	—	—	—
Triphenyl	102.9 ± 1.5	82.0 ± 0.7	92.7 ± 5.8
	(9.2 ± 0.1)	(4.6 ± 0.2)	(4.5 ± 0.7)
Tricresyl	80.9 ± 1.8	101.5 ± 0.1	78.8 ± 4.1
	(5.8 ± 0.2)	(—)	(0.4 ± 0.5)

[a] Two determinations.
[b] Four determinations.
[c] Values in parentheses are for recoveries from a second XAD cartridge when used in series with the first cartridge.

ability of the resin to adsorb low concentrations of organics from a large volume of water. Because it is impractical to collect and fortify 200-L bulk samples of water, a new technique was used to continuously fortify large volumes of tap water with trace levels of phosphate esters. A concentrated aqueous phosphate solution was introduced, using an LC pump, directly into the pressurized (about 80 psi) drinking water line. The flow rates were adjusted to give concentrations of 1, 10, or 100 ng/L before passage through the XAD resin. For all recovery runs, a second cartridge was connected in series to adsorb any unretained phosphate.

Results from Table 2 indicate recoveries similar to the direct method, although some exceptions were noted. Most of the tri(2-ethylhexyl)phosphate, together with small amounts of tricresyl phosphate, were adsorbed by the LC pump, presumably by a small cellulose membrane filter in the pump. Also, the tap water contained a relatively high level of tributoxyethyl phosphate which interfered with quantitation of this compound. Source of the phosphate is the water itself although the 2 rubber O-rings and a rubber seal in the tap may have contributed. Concentrations of tributoxyethyl phosphate in the O-ring and seal were about 5000 and 500 ppm, respectively. Analysis of a grab sample of stale tap water (first 2 L) showed about 6 μg/L tributoxyethyl phosphate which quickly decreased to 200 ng/L by flushing about 30 L water.

The overall results compare well with the direct fortification method, thereby demonstrating a feasible method for the introduction of low levels of organic compounds into large volumes of drinking water for fortification studies. Also, the results demonstrate that the direct spiking approach can provide an approximate measure of recovery efficiency for low level organic compounds from large volumes of water.

Because of the widespread use of phosphate esters as plasticizers and flame retardants, it was necessary to purify all chemicals and solvents and to carefully clean all glassware and apparatus used in the analytical method. Because trialkyl/aryl phosphates have been identified in distilled water (11, 12), the purified water used in the present study to rinse resin cartridges was judged satisfactory only after passing Millipore Super-Q system water through XAD resin. Other potential sources of contamination were glass wool (trixylenyl phosphate); methylene chloride (tributyl phosphate, tributoxyethyl phosphate, triphenyl phosphate, tricresyl phosphate, and 2-ethylhexyl diphenyl phosphate); and Buna-N rubber O-ring (tributoxyethyl phosphate) (Table 3). Replacement of the rubber O-ring from the metering valve with a Viton® O-ring eliminated this possible source of contamination.

Water extracts collected during June and October 1978 were analyzed by GC-NPD for triethyl, tributyl, tris(2-chloroethyl), tri(1,3-dichloropropyl), tri(2-ethylhexyl), tributoxyethyl, triphenyl, and tricresyl phosphates. Analyses were carried out on an OV-17 column and confirmed on an OV-101 column and by GC/MS analyses by searching reconstructed mass chromatograms for at least 2 characteristic ions for the sample and standard within the re-

Table 3. Potential laboratory sources of trialkyl/aryl phosphate contamination

Source	*n*-Butyl	1,3-Dichloropropyl	Butoxyethyl	Phenyl	Cresyl	Others[a]
Super-Q water			120 ng/L			
Corning water		26 000 ng/L		5,900 ng/L		isopropylphenyl diphenyl
Glass wool						trixylenyl
O-ring rubber (metering valve)			10 070 μg/g			
O-ring (Super-C cartridge)					151 μg/g	
Gasket rubber Corning still					576 μg/g	
Methylene chloride	+++		+++	+++	+++	2-ethylhexyl diphenyl

[a] Tentatively identified by comparison with industrial fluids.

tention time window established for the standard. The GC-NPD response for the phosphates studied was linear over the range 0.1–100 ng injected, which covers the range of phosphate concentrations found in tap water. Table 4 lists the retention times of some trialkyl/aryl phosphates on the 2 packed columns used under conditions described in the apparatus section. The table also shows the characteristic ions scanned for GC/MS confirmation.

Extracts were analyzed only for phosphates for which standards were available, and contain several low concentration, unidentified compounds as evidenced by a typical water extract chromatogram (Figure 2b). Figure 2a is a chromatogram of a standard mixture of trialkyl/aryl phosphates.

Because of the complexity of chromatograms of drinking water extracts and the multicomponent nature of many trialkyl/aryl phosphate industrial fluids, analyses were also attempted by capillary column GC. Figure 3, illustrating

Table 4. GC and GC/MS data for some trialkyl/aryl phosphates

	Retention times		GC/MS	
Phosphate	OV-17	OV-101	Selected ions	
Triethyl	20	45	111	182
Tributyl	130	304	155	211
Tris(2-chloroethyl)	310	386	143	249
Tri(2-ethylhexyl)	749	955	113	211
Tris(1,3-dichloropropyl)	770	793	191	381
Tributoxyethyl	798	880	125	299
Triphenyl	863	844	77	326
IPDP[a,b]	949	976	251	368
	1028	1055		
	1091	1136		
Tri-o-tolyl	990	1036	91	368
Tri-*m*-tolyl	1101	1117	91	368
Tri-*p*-tolyl	1225	1291	91	368
Tricresyl	1101	1117	91	368
	1136	1159		
	1176	1204		
	1225	1291		
Tri(2,4-xylenyl)	1432	1531		
Trixylenyl[b]	1275			
	1350			
	1432			
	1524			
	1615			

[a] IPDP = isopropylphenyl diphenyl phosphate.
[b] From industrial fluids.

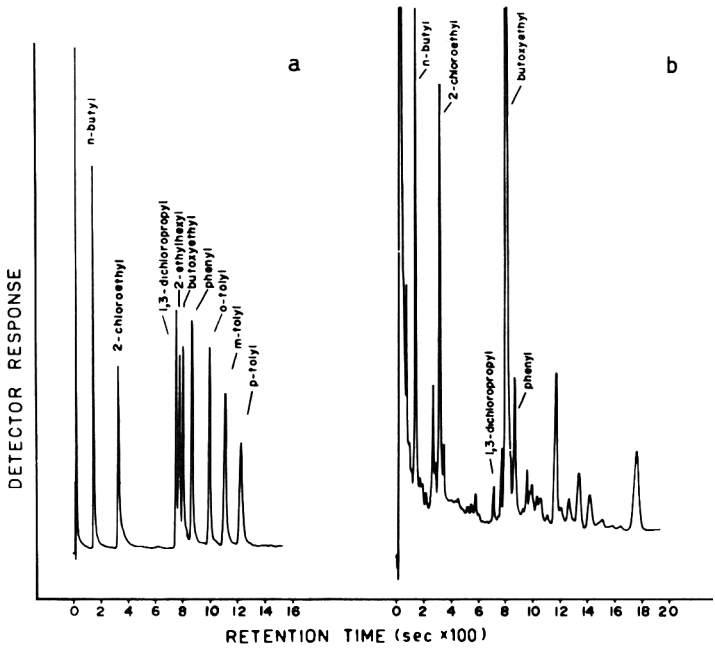

Figure 2. GC-NPD chromatogram on OV-17 column of: (a) 1 ng each of tributyl, tris(2-chloroethyl), tri(2-ethylhexyl), tris(1,3-dichloropropyl), tributoxyethyl, triphenyl, and tri-*o*-, tri-*m*-, and tri-*p*-tolyl phosphates; (b) extract of 203 L tap water (Prescott).

a mixture of trialkyl/aryl phosphates, clearly shows the resolution of the various phosphates, notably the isomers of tricresyl phosphate. This superior resolution should ease the interpretation of an actual drinking water extract (Figure 4) compared to interpretation of the same extract on a packed column (Figure 2b).

Table 5 lists levels of trialkyl/aryl phosphates

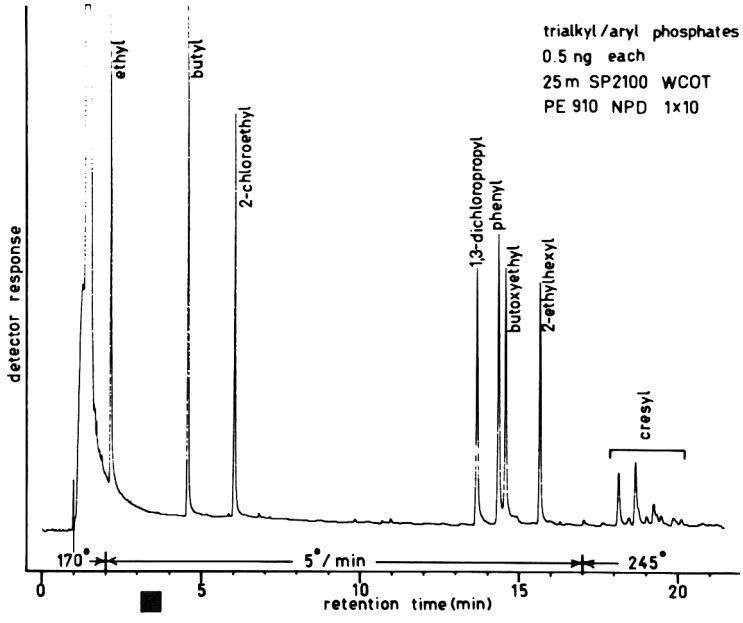

Figure 3. GC-NPD chromatogram of trialkyl/aryl phosphates on 25 m SP2100 WCOT column.

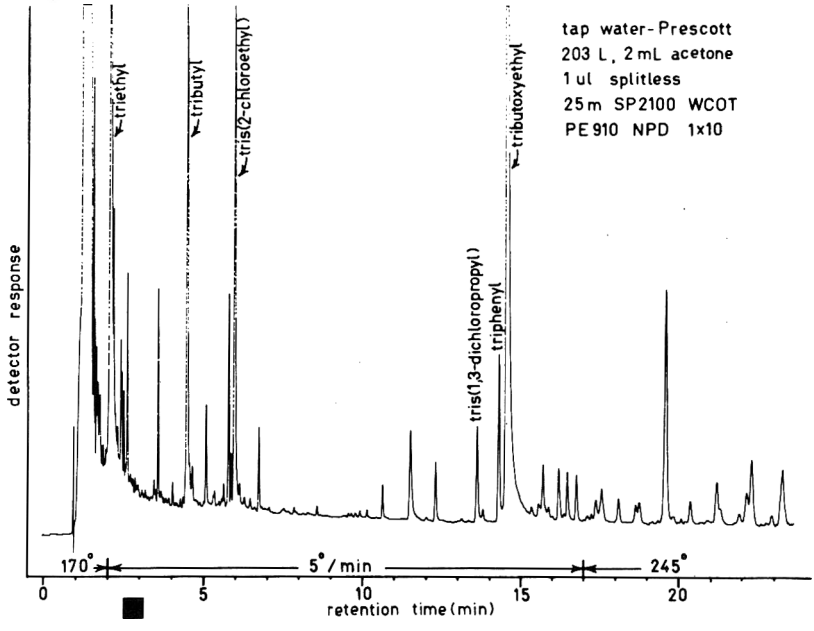

Figure 4. GC-NPD chromatogram of 203 L tap water extract (Prescott) on 25 m SP2100 WCOT column.

found in drinking water from 6 eastern Ontario water treatment plants. The results were not corrected for incomplete recoveries; therefore, triethyl phosphate, where detected, was likely present at a higher concentration. Recovery studies for tris(1,3-dichloropropyl)phosphate at the 1 ng/L level showed recoveries of 100 ± 2%.

In conclusion, a method developed earlier (10) has been shown to be applicable to the determination of trialkyl/aryl phosphates.

A novel approach for fortifying large volumes of drinking water containing very low concentrations of phosphates, using an LC pump, has been applied successfully for recovery studies.

Recoveries were attempted on a few selected phosphates which included trialkyl, trichloroalkyl, and triaryl phosphates. Thus the method should be applicable to the whole class of trialkyl/aryl phosphates.

Acknowledgments

We thank Ron O'Grady, Pauline Lee, and Georgina Griffith for technical assistance.

Table 5. Trialkyl/aryl phosphates (ng/L) in drinking water from 6 eastern Ontario water treatment plants, 1978

Plant	Date	Tri-ethyl	Tri-butyl	Tris-(2-chloro-ethyl)	Tris(1,3-dichloro-propyl)	Butoxy-ethyl	Tri-phenyl	Tri-cresyl	Tri(2-ethyl-hexyl)
Lemieux Is.	Oct		1.7	0.4	0.2	48.9	0.4		0.3
	Oct[a]		1.2	0.3	0.2	70.4	0.4		0.3
Britannia	Oct		0.6	0.4	0.3	8.4	0.7		
	Oct[a]		0.6	0.4	0.2	6.2	1.9		
Smiths Falls	June		1.0	1.0	0.5	2.6	0.2		
	Sept		0.9	1.6	0.7	0.9	0.4	0.3	
Perth	June		0.5	0.7	0.2	3.2	0.3		
	Sept		1.4	1.2	0.2	4.3	0.5		
Prescott	June	24.0	11.2	6.6	1.6	16.2	2.0		
	Oct	19.9	9.8	8.2	1.4	75.4	2.6		
Brockville	June	17.2	11.8	6.9	1.4	8.4	1.2		
	Oct	27.1	11.0	9.2	1.8	10.1	0.7		

[a] Water from raw water tap at treatment plant.

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High Performance Size Exclusion Chromatograph with Computerized Data Reduction for Analysis of Polyethylene and Oligomers

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A liquid chromatograph is described for the size exclusion chromatography (SEC) of polyethylene and oligomers. The rigid column packing material was 10 μm diameter porous silica. An infrared spectrophotometer was used as the detector. The chromatograph was designed and operated to minimize the degree of chromatographic peak shape distortion resulting from factors such as system dead volume, detector cell design, sample injection volume, and sample concentration. To compensate for peak spreading and skewing, the chromatographic data were analyzed with an IBM 370/168 computer using an APL program. The molecular weight range covered by this SEC system was 100-500 000. Precision and accuracy of the calculated weight average molecular weight ($\bar{M}w$) values for polyethylene standards over this range were ±3 and ±4%, respectively.

The Food and Drug Administration (FDA) is responsible for assuring that food packaging materials are safe for their intended use. One aspect of this regulatory activity concerns oligomers which may migrate from polymeric packaging material into food. Our laboratory was initially interested in the oligomers of polyethylene, the polymer most widely used for food packaging. A method capable of yielding an accurate molecular weight distribution (MWD) of food grade polyethylene and its oligomers was required. Size exclusion chromatography (SEC) was chosen to obtain this information.

Over the past 20 years, SEC has been a useful but tedious technique for determining the MWD of a polymer (1–6). A series of 1 m columns was usually employed, requiring several hours for total elution of the sample. Recent advances in column packing technology, however, have given rise to a new class of high performance SEC columns (7). These new packing materials allow analysis by SEC in 15–20 min, using 1 or two 25 cm columns. Increased availability of computers for scientific data handling has further facilitated the development of SEC as a rapid technique for analyzing polymeric materials (4, 8–12).

A liquid chromatograph and computerized data handling capabilities, developed for SEC of polyethylene and oligomers, are described in this paper. The results obtained using this system for the molecular weight (MW) characterization of a number of commercially available food grade polyethylenes and their hexane and xylene extractable oligomeric fractions are reported in a separate paper (13).

The MWD of a polymer analyzed by SEC is determined from an examination of the shape of the chromatographic peak recorded. Peak spreading and skewing, which occur in all chromatographic systems, are therefore detrimental to obtaining an accurate MWD by SEC (14–16). This effect was of particular concern in this work since the greatest degree of peak distortion occurred at the low MW tail of the chromatogram. To minimize this effect, the design and operation of the liquid chromatograph were optimized with regard to system dead volume, detector cell volume, flow rate, column effects, sample size, temperature, and sample concentration (17–23).

The SEC data obtained for each polymer were analyzed using an IBM 370/168 computer. The APL computer program developed for this analysis compensated for peak spreading and skewing. It also performed the voluminous mathematical calculations required to obtain the MWD of the sample. Accurate assignments of retention volumes were ensured through use of an internal standard, 2-*n*-propylphenol (OPP).

Experimental

Reagents

Standards used to calibrate the SEC columns were polyethylene Standard Reference Materials (SRMs) 1475, 1476, 1482, 1483, and 1484, available from the National Bureau of Standards, U.S. Department of Commerce, Washington, DC. Several normal alkanes (purity 99%) obtained from Analabs were also used in the calibration. Reagent grade 1,2,4-trichlorobenzene (TCB) from Eastman Kodak Co. was the mobile phase. OPP (98% purity), used as the internal standard, was obtained from Aldrich.

Apparatus

The solvent delivery pump was a Spectra-Physics Model 740B operated in the constant volume mode. A 6-port valve designed to withstand temperatures to 150°C at 2000 psi, available from Valco Instruments Co., was used

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for sample injection. The columns and valve injector were maintained at 135°C in a modified Spectra-Physics 3400B oven. (Oven modifications: temperature controller electronics adjusted to allow operation to 150°C; portion of oven wall removed to allow mounting injector valve inside oven.) The oven and detector were connected with an 8 in. double insulated transfer line (0.025 mm Teflon tubing). The infrared (IR) detector, equipped with a heated cell holder with an operating temperature range from ambient to 200°C, was obtained from DuPont Instruments. A 1.5 mm path length micro flow-through cell available from Foxboro/Wilks was used as the detector cell. Operating conditions of the IR detector were: cell temperature 145°C, wavelength setting 3.4 μm, slit width 2 mm, meter time constant 4 s, range 0.1 absorbance unit full scale deflection (AUFSD) (1 mV). The refractive index (RI) detector was a Waters Associates Model R401. A Haake Model FE circulating water bath was used to maintain the RI cell at 75°C. The SEC columns were obtained from DuPont Instruments (SE-500, SE-100, and SE-60) and Waters Associates (100, 500, and 1000 Å μStyragel). A Hewlett-Packard Model 7130A variable range/speed strip chart recorder was used to record the detector output. Chromatographic area slicing was achieved with a Spectra-Physics Minigrator operated in the simulated distillation mode. The area slice data received from the Minigrator were subsequently analyzed with an IBM 370/168 computer.

Intrinsic viscosities were measured in a Hewlett-Packard 5910A constant temperature bath operated at 135°C. Two Cannon-Ubbelohde semimicro dilution viscometer tubes (sizes 25 and 50) were used, with TCB as the solvent.

Procedures

The samples and standards were prepared as 0.1–0.5% solutions in TCB containing 0.2% of the internal standard (OPP). They were dissolved by stirring on a hot plate ca 1 h at 150°C. The hot, unfiltered solutions were then transferred to the loop injector with a hot 500 μL syringe.

Intrinsic viscosities were determined by measuring the inherent viscosity (at 135°C) at several concentrations (in TCB) and extrapolating to zero concentration (24).

Results and Discussion

System Dead Volume

Dead volume, located in a liquid chromatograph between the injector and the detector, is known to cause chromatographic peak spreading and skewing (17–21). All transfer lines in the chromatograph therefore were made with 0.025 mm id tubing and all connections were made with zero dead volume connectors. The total system dead volume due to tubing and connectors was kept to approximately 70 μL by minimizing the lengths of these transfer lines.

Injection Volume and Sample Concentration

Chromatographic peak broadening is also a function of sample injection volume (22). The objective was to select an appropriate volume which minimized peak distortions, while introducing sufficient sample to allow detection with adequate sensitivity. Obviously, increasing the sample concentration decreased the volume required, but this was feasible only within certain limits (22, 23). In this study, distorted peaks, which sometimes appeared as doublets, were encountered when sample concentrations of about 1% or greater were injected. Therefore, sample concentrations were limited to between 0.2 and 0.5% throughout this work.

An injection volume best suited to this system was determined by comparing the resolution observed for decane and eicosane at 10, 50, 100, and 200 μL. Optimum resolution and sufficient sensitivity were obtained with a 50 μL sample loop.

Choice of LC Detector

In selecting the optimum detector, both RI and IR detectors were examined. A detector was required that provided adequate sensitivity for polyethylene and a stable baseline when operated at 135°C.

The RI detector examined contained a micro flow-through cell (20 μL effective volume) with an upper temperature limit of 75°C. In determining the sensitivity of this detector, the limiting factors were baseline instability and high frequency noise. Also, due to RI dependence on MW, the detector response had to be calibrated. In light of these difficulties, an IR spectrophotometer was evaluated as an alternative detector.

The variable wavelength IR detector was equipped with a heated flow-through cell having an upper temperature limit of 200°C. The 3.4 μm wavelength, which measured the CH_2 stretching frequency and was therefore independent of MW, was monitored. In establishing this detector's optimum sensitivity, an erratic

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Table 1. Effects on performance due to detector cell design [a]

Detector cell design	Effective cell vol., μL	Sample injection vol., μL	No. theoretical [b] plates (C_{23})	Resolution [c] C_{20} and C_{10}
RI - quartz cell as supplied, 8 μL cell volume plus 12 μL transfer line	20	100	~10 000	0.73
IR - as supplied, oval 40 × 4 × 1 mm cell ~150 μL total volume, IR beam aligned at cell center	100	100	~7000	0.64
Oval 40 × 4 × 1 mm cell in optimized position, IR beam aligned at bottom portion of cell	20	100	~10 000	0.72
Micro flow-through cell, 4 mm diameter, 1.5 mm thick	20	100	~9200	0.72
		50	~11 500	0.81

[a] Conditions: One SE-500 and one SE-60 DuPont size exclusion column; 1 mL/min; TCB; 140°C.
[b] Number theoretical plates for C_{23} = 16 (retention time/width at base)^2
[c] Resolution between C_{20} and C_{10} = 2 (Δ retention time)/(C_{20} width at base + C_{10} width at base).

baseline was experienced when it was operated at its maximum sensitivity of 0.025 AUFSD. Sensitivity superior to that achievable with the RI detector was obtained, however, at 0.1 AUFSD and a meter time constant of 4 s. Under these conditions the IR detector response time was very good, with minimal peak shape distortion. Baseline stability and high frequency noise were also excellent with a cell temperature of 135°C.

Detector Cell Design

Initial replacement of the RI detector with the IR detector resulted in a dramatic reduction in resolution. This loss in resolution was due to the detector cell design. The cell supplied with the IR detector was 40 × 4 × 1 mm, with a total volume of about 150 μL. The beam of the IR detector, however, was only 4 mm × 2 mm (0.5–2.0 mm wide depending on the slit width selected) and directed at the center of the cell. The sample entered the bottom of the cell and before detection at the center traveled through approximately 100 μL cell dead volume. Dynamic mixing occurred and distortion of the chromatographic band resulted. Repositioning the cell so that the IR beam was directed at the inlet portion of the cell minimized this effect (Table 1). The sample eluting from the top of the cell was of course still badly distorted and unacceptable if fraction collection was desired.

To further optimize the IR detector, a Foxboro/Wilks micro flow-through cell was investigated. This cell consisted of a stainless steel plate with a circular 2 mm diameter cell compartment, 1.5 mm thick, covered with NaCl windows, and with a volume of 4.7 μL. Although very good resolution was obtained, an excessive baseline noise level resulted when the system was operated at 0.1 AUFSD. This was due to the 2 mm cell diameter, relative to the 4 mm IR beam length. To alleviate this problem, the stainless steel plate of the cell was drilled to a 4 mm diameter cell compartment, increasing the cell volume to about 20 μL. This final cell design optimized resolution of the chromatographic peaks (Table 1) and due to its 1.5 mm vs 1.0 mm pathlength yielded an overall increase in sensitivity.

SEC Column Packing Material

Several basic criteria needed to be met in selecting the SEC columns for this study. First, the column packing had to be capable of withstanding temperatures of 130–150°C when it was in contact with the TCB required for solution of polyethylene. Second, the column's calibration of MW vs retention volume had to be linear and cover the MW range of the polymer samples. A third requirement was that the columns have sufficient resolving power to enable characterizing the oligomers present in the polyethylene extracts (MW ≤5000). The fourth requirement was that the analysis time be several minutes and not several hours. This requirement had to be achieved at a flow rate compatible with the resolution requirement and at a column back pressure of less than 2000 psi, the operating limit of the valve injector.

Initial studies were conducted using a series of μStyragel columns (100, 500, and 1000 Å). These columns performed adequately in terms of: (*1*) overall linear column calibration, (*2*) resolving power, and (3) speed of analysis. However, the columns (especially the 100 Å column) were not compatible with TCB at a flow rate of 1 mL/min at 130°C. Therefore, a different SEC packing material was sought.

The rigid porous silica SEC packing materials which might be better suited for the rigorous operating conditions required were examined. DuPont Instruments' size exclusion SE series

porous silica columns, 10 μm particle diameter and 60, 100, and 500 Å (SE-60, SE-100, and SE-500, respectively) pore diameters, were chosen. Several different DuPont SEC columns were examined in selecting the optimum combination to meet the requirements specified above.

Excellent resolving power for the oligomers of polyethylene was observed when 2 SE-60 columns were combined. However, the upper MW limit for these SE-60 columns was only 4000. Combination of one SE-60 column with one SE-100 column extended this limit to approximately 35 000 MW, but this still was inadequate for the analysis of most polyethylene resins. Also, the column calibration for one SE-60 column plus one SE-100 column was not linear over the entire MW range (100–34 000), but rather consisted of 2 separate curves joined together with a discontinuous step around 1000 MW. This effect has previously been reported (16) and is attributed to the degree to which the MW ranges covered overlap.

The column pair ultimately selected was a combination of one SE-60 column and one SE-500 column with MW ranges of about 100–4000 and 4000–500 000, respectively. The MW vs retention volume calibration curve for this column pair was continuous over its entire MW range and covered the range of MW present in most of the polyethylene samples. The resolving power for these columns also met the requirements regarding the separation of the low MW oligomers of polyethylene. Furthermore, a typical analysis could be performed in about 15 min at a flow rate of 1.0 mL/min and a column back pressure of about 400 psi. All 4 of the requirements outlined above for the SEC columns therefore were satisfied by this column pair.

Column Calibration

MW vs retention time calibration of the SEC columns was initially established using several well characterized linear polyethylene standards (SRMs 1475, 1482, 1483, and 1484) and *n*-alkanes (dotriacontane and undecane). An equation that best describes this calibration is:

$$\text{Log M} = A + BT + C(T')^5 \qquad (1)$$

where M is molecular weight, T is retention time (s), A is 9.330, and B is −0.0127. The third element, $C(T')^5$, compensated for the nonlinearity occurring near the void and total permeation volumes ($C = 0.00655$, $T' = 440 - T$).

Because this work entailed both low density (branched) and high density (linear) polyethylene, a universal calibration was desired. This was accomplished, as has been done by others (9, 25–35), using the intrinsic viscosity-MW relationship which for high density polyethylene is described by:

$$\text{Log } [\eta] = \log K + \alpha \log \text{M} \qquad (2)$$

where $[\eta]$ is intrinsic viscosity. Several values have been reported (25–28) for α and K, and we found $K = 0.0004$ and $\alpha = 0.73$ to yield the best fit of our data.

For low density polyethylenes, this intrinsic viscosity MW relationship is a function of the degree of branching present, and expanded versions of Equation 2 have been suggested (28, 29). Wagner and McCrackin (27), for example, represented this relation with a second order polynomial expression. In our work, the same values for α and K were applicable for both linear and branched polyethylene if the following expression was used:

$$\text{Log } [\eta] = \log K + \alpha \log \text{M} + \beta ((\log \text{M}')^4) \qquad (3)$$

($K = 0.0004$, $\alpha = 0.73$, $\beta = -0.0062$ for branched, $\beta = 0.0$ for linear, and $\text{M}' = \text{M} - 6000$ but always ≥ 1). The threshold value of 6000 MW used in defining M′ was chosen based on the findings of Ram and Miltz (28) and other workers (27, 30). The dependence of intrinsic viscosity on MW is a function of the degree of long-chain branching which generally decreases (approaching zero) with decreasing MW (down to about 6000). This accounts for the negligible difference in intrinsic viscosity for branched and linear polyethylenes of MW less than 6000. This low MW fraction of the polymer may contain a relatively high degree of short-chain branching (31, 32). However, the effect due to these short-chain branches was calculated by Drott and Mendelson (29) to have only about 1% the effect that long-chain branches have on the intrinsic viscosity.

In this study when Equation 3 was used to describe this intrinsic viscosity-MW relationship, the same β value was applied to all low density polyethylenes analyzed. A certain degree of error, therefore, resulted since each low density polyethylene probably contained a different degree of long-chain branching. Ideally, a different β value should have been used for each different polymer analyzed. The major objective of this work, however, was to accurately describe the low MW materials in food grade polyethylenes. Since β had no effect on MW <6000, use of an average β value was deemed adequate.

Combining Equations 1 and 3 then yielded the final calibration equation of retention time vs

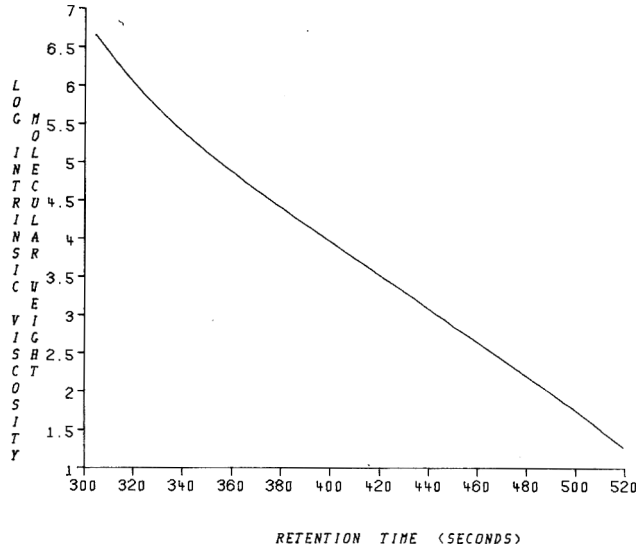

Figure 1. Log MW × intrinsic viscosity vs retention time for one SE-500 and one SE-60 DuPont size exclusion columns.

MW as a function of intrinsic viscosity (Figure 1):

$$\text{Log}([\eta]\text{M}) = \log K + (1+\alpha)(A + BT + C(T)^5) + \beta((A + BT + C(T')^5) - 3.78)^4 \quad (4)$$

for both linear ($\beta = 0$) and branched ($\beta = -0.0062$) polyethylenes.

Internal Standard

The flow rate precision of the solvent delivery pump was ±0.5%. Daily flow rate differences, however, varied as much as ±5%, due to the ambient temperature dependence of the pump's flow controller. With a working volume of approximately 6 mL (total permeation volume minus void volume) for the SEC column pair, these flow fluctuations were of significant concern. Therefore, accurate assignments of retention volume required correcting for these deviations, which was accomplished by using an internal standard.

OPP was selected as the internal standard because it was slightly adsorbed by the SEC column packing material. Therefore, it eluted after the total permeation volume and did not interfere with the analysis. Use of OPP also allowed simplification of daily operating procedures, since frequent system recalibration was not necessary. This made it practicable to expend greater effort in deriving a more accurate column calibration.

Computer Data Handling

Design and operation of the size exclusion chromatograph minimized chromatographic peak distortion but did not completely eliminate this problem. A computer program therefore was written which compensated for these distortions and performed the necessary calculations required to obtain the MWD.

As is usual in SEC, the chromatographic peak obtained for each polymer was divided into area slices of equal width. This was accomplished by connecting the detector output to an integrator operated in the simulated distillation mode. A slice width of 4 s was chosen throughout this study; for most polymers this resulted in dividing the recorded SEC peak into between 50 and 70 area slices. These raw area slice (A_i) vs retention time (T_i) data were analyzed with an IBM 370 computer using an APL program. The computer program performed 6 tasks: (*1*) corrected for baseline shifts, (*2*) corrected for flow rate variations, (*3*) corrected for peak shape distortions, (*4*) assigned a MW to each area slice according to its retention time, (*5*) performed all mathematical calculations, and (*6*) formatted and printed the results.

Corrections for Baseline Shifts and Flow Rate Variations

The integrator used to obtain the area slice measurements did not correct for any baseline

shifts which may have occurred. Adjustment for baseline shifts, therefore, was accomplished with the computer by projecting a straight line between the peak start and end points. The start and end points assigned to each sample were supplied by the operator after preliminary examination of the raw integrator area slice data. Any flow rate fluctuation was corrected by adjusting the measured retention times for each area slice relative to the retention time of the internal standard.

Compensation for Chromatographic Peak Distortion

Chromatographic peak spreading and skewing that occurred during the SEC analysis were observed to be a function of both elution volume and sample dispersity. The correction applied by the computer, compensating for these distortions, therefore was divided into several components. This facilitated determining the appropriate correction to be applied to each individual polymer.

The factor needed to compensate for elution volume-dependent peak distortions was empirically derived and found to be minimal for materials eluting near the mid-range of the SEC columns (about 1000–35 000). Conversely, the greatest correction was needed for samples eluting near the void (about 500 000) and, particularly, the total permeation (about 100) volumes. This component (*F1*) of the total correction for peak distortion was determined by relating the peak maximum retention time (*Tmax*, s) to the midpoint of the column calibration (460 s):

$$F1 = 15\,[0.24 + (|(Tmax - 460)|/150)]^3 \quad (5)$$

With regard to sample dispersity effects, polymers of broad MWD were distorted to a lesser extent than polymers of narrow distribution. A relative measure of this component spreading was obtained by comparing the recorded peak width (*End-Start*) in seconds to the narrowest peak width obtainable (75 s). A correction factor (*F2*), which included compensation for peak distortions due to both MW and dispersity effects was obtained from the following equations:

$$F2 = (75/(End\text{-}Start)) \times F1 \quad (6)$$

Using this correction factor (*F2*), a series of correction factors (C_i) was derived from the retention times (T_i) of the area slice measurements (A_i):

$$C_i = [(|T_i - Tmax|/(End\text{-}Tmax)) \times (F2/2.25)] \quad (7)$$

Multiplying this set of correction factors (C_i) times the area slice data (A_i):

$$CA_i = C_i \times A_i \quad (8)$$

yielded the desired corrected area slice measurements (CA_i), compensated for chromatographic peak spreading and skewing.

Calculation of Molecular Weight Averages

Each corrected area slice (CA_i) according to its retention time (T_i) was assigned a molecular weight (M_i) using Equation 4 (linear $\beta = 0$, branched $\beta = -0.0062$). This equation, however, required first calculating the intrinsic viscosity [η_i] of each area slice (CA_i) and, due to the mathematical nature of Equation 3, was accomplished by an iterative procedure.

Using this set of molecular weights (M_i), the weight average molecular weight ($\overline{M}w$), number average molecular weight ($\overline{M}n$), and dispersity were calculated:

$$\overline{M}w = \Sigma M_i CA_i / \Sigma CA_i \quad (9)$$

$$\overline{M}n = \Sigma CA_i / \Sigma (CA_i / M_i) \quad (10)$$

$$\text{Dispersity} = \overline{M}w / \overline{M}n \quad (11)$$

Similarly, for comparison the uncorrected $\overline{M}w$, $\overline{M}n$, and dispersity were also calculated using the uncorrected area slice measurements (A_i) in place of the corrected areas (CA_i) in Equations 9–11.

Corrected and uncorrected $\overline{M}w$ and $\overline{M}n$ values, together with the reported literature values, for 4 polyethylene standards and 2 *n*-alkanes are listed in Table 2. Also included in Table 2 are the percent standard deviation and the percent error (relative to the literature value) obtained from 5 replicate analyses of each of the 6 standards. The average percent errors (all 30 replicates) for the corrected $\overline{M}w$ and $\overline{M}n$ values were ±3.8 and ±4.2%, compared to ±6.1 and ±23.4% observed for the uncorrected $\overline{M}w$ and $\overline{M}n$. Figures 2 and 3 are the corrected and uncorrected chromatograms obtained for dotriacontane and SRM 1475, respectively. These figures indicate the degree of correction that was applied and its effect on the calculated MWD.

Intrinsic viscosities were measured for 3 low density food grade polyethylene samples (Nos. 1, 5, and 6). The intrinsic viscosity of the low MW portion fractionated from these resins (13) with xylene at 25°C (Samples 5X and 6X) was also determined for 2 of these samples. The intrinsic viscosities of SRMs 1475 and 1476 were also

Table 2. SEC determined molecular weight averages of polyethylene standards

	Uncorrected chromatogram			Corrected chromatogram			
Standard	Av.	SD, %	Error,[a] %	Av.	SD, %	Error,[a] %	Literature
SRM 1484							
$\bar{M}w$	117 670	4.8	−1.6	121 735	1.4	+1.8	119 600
$\bar{M}n$	72 160	13.1	−28.2	97 870	3.9	−2.6	100 500
SRM 1483							
$\bar{M}w$	33 700	3.0	+5.0	33 650	1.9	+4.8	32 100
$\bar{M}n$	23 010	4.3	−20.0	27 000	2.4	−6.6	28 900
SRM 1482							
$\bar{M}w$	13 600	3.4	+0.0	13 650	1.8	+0.4	13 600
$\bar{M}n$	10 685	6.3	−6.3	11 820	3.8	+3.7	11 400
SRM 1475							
$\bar{M}w$	60 820	3.6	+14.6	56 320	4.1	+6.1	53 070
$\bar{M}n$	14 975	20.1	−18.2	19 090	10.1	+4.2	18 310
n-Dotriacontane							
$\bar{M}w$	432	3.6	−1.8	475	2.5	+5.6	450
$\bar{M}n$	355	5.5	−22.0	455	4.1	+1.1	450
n-Undecane							
$\bar{M}w$	135	2.9	−13.5	149	3.5	−4.4	156
$\bar{M}n$	85	28.3	−45.5	145	2.7	−7.0	156
$\bar{M}w$[b]		3.5	6.1		2.5	3.8	
$\bar{M}n$[b]		12.9	23.4		4.3	4.2	

[a] Calculated average relative to literature value.
[b] Simple averages for the 6 standards.

measured for comparison. Table 3 contains the measured intrinsic viscosities as well as the value calculated from the SEC analysis of these samples. Literature values and those calculated for some of the standards are also listed. For the 5 low density polyethylene samples, the agreement between the calculated and the measured intrinsic viscosities using the one β value of −0.0062 is very good. For SRM 1476, however, better agreement between the calculated and the literature intrinsic viscosities could be obtained using a value for β of −0.0043. Therefore the degree of branching appeared to be different for SRM 1476.

All of the linear polyethylene standards showed very good agreement between the calculated and reported intrinsic viscosities. This

Table 3. Intrinsic viscosities of several low and high density polyethylenes[a]

Sample	Reported [η]	Measured [η]	Calculated[b] [η]
Low Density (β = −0.0062)			
1	—	0.89	0.91
5	—	0.80	0.80
6	—	0.68	0.64
5X	—	0.12	0.10
6X	—	0.10	0.12
SRM 1476	0.90	0.88	0.75 (0.90)[c]
High Density (β = 0)			
SRM 1484	1.98	—	2.04
SRM 1483	0.79	—	0.78
SRM 1482	0.40	—	0.41
SRM 1475	1.01	0.98	1.02

[a] Measured in TCB at 135°C, dL/g.
[b] Calculated from the corrected SEC chromatograms.
[c] Using β = −0.0043.

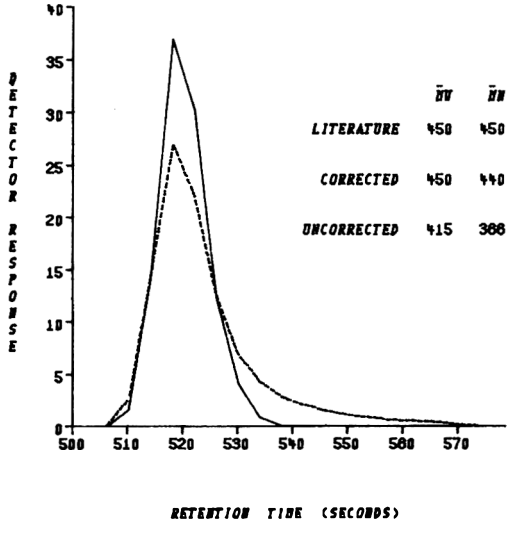

Figure 2. *n*-Dotriacontane corrected (solid line) and uncorrected (dashed line) SEC chromatograms.

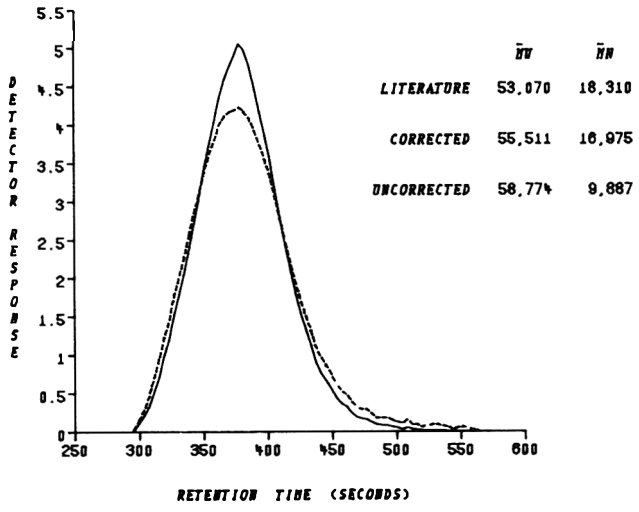

Figure 3. SRM 1475 corrected (solid line) and uncorrected (dashed line) SEC chromatograms.

lends further confidence to both the validity of the α and K values used and the manner in which the computer program compensated the raw area slice data for chromatographic spreading and skewing.

An accurate calibration in the low MW region (<500) was further assured by analyzing a synthetic test mixture composed of a series of *n*-alkanes at known concentrations (Table 4). The theoretical $\overline{M}w$ and $\overline{M}n$ values for this mixture were 339 and 289, respectively. The calculated, uncorrected $\overline{M}w$ and $\overline{M}n$ values obtained from its SEC analysis were 316 and 173, respectively, compared with the computer corrected values of 346 and 289, respectively. The agreement between the actual and computer corrected data is very good.

Table 4. SEC of synthetic test mixture of *n*-alkanes

n-Alkane	MW	Actual wt %	Corrected wt %	Uncorrected wt %
C_6	86	1.3	0.9	3.2
C_8	114	2.4	2.2	4.4
C_{10}	142	3.9	3.3	5.6
C_{12}	170	5.2	4.9	6.7
C_{16}	226	8.5	8.9	9.4
C_{20}	282	13.8	14.5	12.9
C_{23}	324	18.0	18.1	14.5
C_{28}	394	23.3	17.9	14.5
C_{32}	450	16.3	15.0	13.5
C_{36}	506	5.7	10.9	11.2
C_{44}	618	1.6	3.3	3.8
	$\overline{M}w$	$\overline{M}n$	Dispersity	
Theoretical	339	289	1.17	
Corrected SEC	346	289	1.20	
Uncorrected SEC	316	173	1.82	

Conclusion

A high performance liquid chromatographic technique for the SEC of low MW polyethylenes has been described. Through use of an APL computer program, compensation for chromatographic peak shape distortion was achieved. MW data calculated on this system were in good agreement with reported values for a variety of polyethylene standards.

Acknowledgments

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Molecular Weight Characterization of Food Grade Polyethylenes and Extractables by Size Exclusion Chromatography

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Size exclusion chromatography (SEC) was used to characterize the molecular weight distribution (MWD) of 6 low density and 6 high density food grade polyethylene resins. The hexane and xylene extractable fractions of these 12 resins were also analyzed by SEC. An IBM 370/168 computer with an APL program was used to analyze the chromatograms and correct for chromatographic band spreading and skewing. Calculated weight average molecular weights ($\overline{M}w$) for the resins ranged from 40 000 to 200 000 and number average molecular weights ($\overline{M}n$) ranged from 6000 to 60 000. Median values of $\overline{M}w$ and $\overline{M}n$ were 1800 and 950, respectively, for hexane extractables from the low density resins, and were 310 and 290, respectively, for hexane extractables from the high density resins. Corresponding $\overline{M}w$ and $\overline{M}n$ values for xylene extractables were consistently larger than those for hexane extractables.

Many food commodities are packaged in polymeric materials. Components of these polymers have the potential for migrating into the food and becoming indirect food additives. Therefore, food packaging materials must comply with Food and Drug Administration (FDA) regulations regarding the safety of their intended use (1). Of the numerous polymers utilized as food packaging materials, polyethylene is by far the most widely used and accounts for over 60% of the total tonnage of food grade polymers (2). In the original petitions submitted to FDA for approval of food contact use of polyethylene, data were submitted supporting the safety of this use. An FDA regulation was promulgated whereby compliance of marketed food grade polyethylenes could be ensured through the use of 2 specific "end-test" procedures (3), which (a) describe extraction of the polyethylene with either hexane or xylene under specific conditions, and (b) specify allowable limits for the weight percent extractable fraction. These tests do not provide specific knowledge about the chemical identity of the extractable materials, but merely give a relative measure of the weight percent extracted.

The FDA regulations and end-tests for polyethylene were developed nearly 20 years ago. Since that time, manufacturing processes have changed and the fields of analytical chemistry and toxicology have advanced. These changes, as well as the increasing need within FDA for a better understanding of the low molecular weight (MW) materials present in food packaging polymers, led to our reinvestigation of polyethylene, using newly developed analytical techniques. We hoped that the information obtained would contribute to a simplification of the requirements of current and future petitions for regulated food contact use of polyolefin type materials.

The scope of the study entailed characterizing the molecular weight distribution (MWD) of extracts obtained from food grade polyethylene resins by the hexane and xylene end-test procedures. The oligomers extracted by these end-test procedures were not necessarily identical to the material migrating under actual use conditions. The end-test procedures, however, proved to be a convenient means for isolating low MW oligomers. The low MW fraction could then be analyzed separately from the whole polymer, and a more detailed description of its MWD was obtained.

The polyethylene resins examined were obtained from 3 of the leading manufacturers of food grade polyethylene. Each manufacturer supplied 2 low density and 2 high density resins that are currently marketed. These 12 resins were considered to be representative of the types of food grade polyethylenes now being sold.

Size exclusion chromatography (SEC) was chosen for characterization of these resins and their extracts because the MWD of a polymer can be obtained by this technique (4-11). The SEC system developed was capable of characterizing both low and high density polyethylenes, and was designed to place special emphasis on the ability to analyze the low MW materials that were fractionated from the whole resins with either hexane or xylene in the FDA polyethylene end-test procedure. A separate paper (12) reports details about the size exclusion chromatograph and an explanation of the corrections that are applied during the computerized data handling to compensate for chromatographic peak distortions.

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Experimental

Apparatus

The chromatographic system consisted of a Spectra-Physics Model 740B solvent delivery pump operated at 1.0 mL/min, a DuPont Instruments infrared spectrophotometer (wavelength 3.4 μm, pathlength 1.5 mm), a 6-port, loop injector valve from Valco Instruments designed for operation to 150°C, a 50 μL sample loop, and a Spectra-Physics oven modified to operate at 145°C. The SEC columns, one SE-500 and one SE-60 (DuPont Instruments), contained 10 μm porous silica with nominal pore diameters of 500 and 60 Å, respectively. Chromatographic peaks were integrated with a Spectra-Physics Minigrator operated in the simulated distillation mode. Area slice measurements received from the Minigrator were processed by an IBM 370/168 computer using an APL program (12). A Tektronix Graphics display terminal with a modified version of APL GRAPH 2 software was used to prepare all figures.

Reagents

Standards used to calibrate the SEC system included polyethylene Standard Reference Materials (SRMs) 1475, 1476, 1482, 1483, and 1484, available from the National Bureau of Standards, U.S. Department of Commerce, Washington, DC, and several *n*-alkanes (99% purity), available from Analabs. The mobile phase was reagent grade 1,2,4-trichlorobenzene from Eastman Kodak. Hexane (distilled in glass) and xylene (ACS grade) used in the extraction studies were obtained from Burdick and Jackson and from Fisher Scientific, respectively. 2-*n*-Propylphenol (OPP, 98% purity), used as the internal standard, was obtained from Aldrich.

Procedures

All 12 resins were extracted with both hexane and xylene by the procedures outlined in the *Code of Federal Regulations*, Title 21, Part 177.1520(d)(3)(ii) and (4)(ii) (3). Before extraction, each resin was ground (20–40 mesh) in a Wiley mill. Hexane extractables were obtained by stirring a 2.5 g portion of the ground resin for 2 h in 1 L hexane at 50°C, filtering the hot mixture, and evaporating the filtrate to dryness in a tared flask. Xylene extractables were obtained by stirring 5 g ground resin in 1 L refluxing xylene (140°C) for 2 h, which completely dissolved the resins. The solution was cooled to 25°C, which reprecipitated most of the polymer, and filtered. The filtrate was then evaporated to dryness in a tared flask.

For SEC, the whole polymers and the extracts were prepared as 0.5% solutions in 1,2,4-trichlorobenzene. The internal standard, OPP, was added at a concentration of 0.25% and solution was achieved by heating on a hot plate at 140°C for ca 1 h. The hot, unfiltered sample was then transferred to the 50 μL sample injector loop of the size exclusion chromatograph and injected. The chromatographic area slice data obtained were manually entered into an IBM 370/168 computer. An APL computer program was used to calculate the MW averages and report a detailed listing of the overall MWD.

Results and Discussion

Calibration of the Size Exclusion Chromatograph

Accurate assignments of retention volumes are of major concern when working in SEC and were ensured through use of an internal standard, OPP. This compound was selected because it was slightly adsorbed by the SEC column packing material and eluted after the total permeation volume; consequently it did not interfere with the MW calculations. The numerous mathematical calculations required for determination of the weight average molecular weights ($\overline{M}w$) and number average molecular weights ($\overline{M}n$) were performed with an IBM 370/168 computer. The APL computer program also corrected the observed chromatograms for band spreading and skewing, and made the necessary adjustments for fluctuations in flow rate indicated by the internal standard. The SEC columns were calibrated by analyzing several well characterized polyethylene SRMs and a series of linear alkanes. The MW range of the standards was from 156 to 110 500 (peak maximum MW). Average differences observed between the calculated $\overline{M}w$ and $\overline{M}n$ values and the reported literature values for the 6 standards listed in Table 1 were ±3.8 and ±4.2%, respectively. The average relative standard deviation for these calculated $\overline{M}w$ and $\overline{M}n$ values were ±2.5 and ±4.3%, respectively. The derivation of this calibration is discussed in ref. 12.

Accuracy in the very low MW range was further assured by analyzing a mixture comprised of a series of *n*-alkanes (C_6, C_8, C_{10}, C_{16}, C_{20}, C_{23}, C_{28}, C_{32}, C_{36}, and C_{44}), each at known concentrations. The theoretical $\overline{M}w$ and $\overline{M}n$ values for this mixture were 339 and 289, respectively, and calculated values obtained from its SEC analysis

Table 1. SEC calculated molecular weight averages of 6 linear polyethylene standards

Standard	Literature	Experimental		
		Av.	RSD, %	Error, %
SRM 1484				
$\overline{M}w$[a]	119 600	121 735	1.4	+1.8
$\overline{M}n$[b]	100 500	97 870	3.9	−2.6
SRM 1483				
$\overline{M}w$	32 100	33 645	1.9	+4.8
$\overline{M}n$	28 900	27 000	2.4	−6.6
SRM 1482				
$\overline{M}w$	13 600	13 650	1.8	+0.4
$\overline{M}n$	11 400	11 820	3.8	+3.7
SRM 1475				
$\overline{M}w$	53 070	56 320	4.1	+6.1
$\overline{M}n$	18 310	19 085	10.1	+4.2
n-Dotriacontane C-32				
$\overline{M}w$	450	475	2.5	+5.6
$\overline{M}n$	450	455	3.1	+1.1
n-Undecane C-11				
$\overline{M}w$	156	149	3.5	−4.4
$\overline{M}n$	156	145	2.7	−7.0
$\overline{M}w$[c]			2.5	±3.8
$\overline{M}n$[c]			4.3	±4.2

[a] $\overline{M}w = \Sigma(C_iMW_i)/\Sigma C_i$.
[b] $\overline{M}n = \Sigma C_i/\Sigma(C_i/MW_i)$.
[c] Simple average for the 6 standards listed.

were 346 ($\overline{M}w$) and 289 ($\overline{M}n$), showing excellent agreement.

Size Exclusion Chromatography of Food Grade Polyethylenes

The calculated $\overline{M}w$ and $\overline{M}n$ values for the 12 food grade polyethylene resins characterized by SEC, together with the manufacturer's reported melt index and density information, are listed in Table 2. The melt index, a measure of polymer flowability in g/10 min determined according to ASTM Method D1238 (13), tended to increase with decreasing polymer MW. No apparent relationship was evident between MW and density of the resin.

The $\overline{M}w$ values for the 6 low density resins ranged from ~80 000 to ~200 000 and the $\overline{M}n$ values ranged from ~6000 to ~60 000. For the 6 high density resins, these ranges were $\overline{M}w$ ~40 000 to ~140 000 and $\overline{M}n$ ~13 000 to ~40 000.

Table 2. Density, melt index, and molecular weight averages for 12 food grade polyethylene resins

Sample	Density, g/mL[a]	Melt index, g/10 min[a,b]	$\overline{M}w$	$\overline{M}n$	Dispersity ($\overline{M}w/\overline{M}n$)
		Low Density Resins			
1	0.918	2.0	198 700	23 900	8.3
2		1.0	197 300	59 400	3.3
3	0.920	0.25	204 000	47 850	4.3
4	0.924	23.0	87 190	6 130	14.2
5		23.9	147 100	25 710	5.7
6	0.923	35.0	82 870	18 560	4.5
		High Density Resins			
7	0.953	0.1	139 590	18 660	7.5
8		1.1	132 000	40 060	3.3
9	0.965	0.6	92 050	13 310	6.9
10	0.953	4.0	75 810	17 210	4.4
11		18.2	61 280	23 200	2.6
12	0.960	42.0	41 630	17 060	2.4

[a] Data supplied by manufacturers.
[b] Melt index determined according to ASTM method D1238 (13).

Table 3. Molecular weight distribution data for polyethylene hexane extractables

Sample	Wt % extractable[a]	$\overline{M}w$	$\overline{M}n$	Dispersity ($\overline{M}w/\overline{M}n$)
		Low Density Resins		
1	4.5	2832	1243	2.3
2	0.5	1683	817	2.1
3	0.7	1628	895	1.8
4	3.0	1933	76	25.3
5	2.2	2540	1510	1.7
6	1.0	1510	978	1.5
Median	1.6	1808	936	
		High Density Resins		
7	0.1	308	264	1.2
8	0.01	315	292	1.1
9	0.6	309	288	1.1
10	1.1	330	290	1.1
11	0.2	293	254	1.2
12	0.1	326	291	1.1
Median	0.15	312	289	

[a] Determined after hexane extraction procedure. Allowable limit 5.5%.

The averages show that these 12 resins cover a diverse sampling of food grade polyethylenes, both in MW and sample dispersity.

Hexane Extractable Material

A definite difference existed in the percentage of material extractable by hexane from the high and the low density polyethylene resin (Table 3). The median percent extracted by hexane from the 6 low density polyethylenes was 1.6 compared with 0.15 from the 6 high density resins. The percent extractables allowed by the current regulation is 5.5 regardless of density (3).

SEC of the hexane extractable residues provided an understanding of their MWD. Table 3 lists the calculated $\overline{M}w$ and $\overline{M}n$ values for these residues, and the relationship between the density of the polyethylene resin extracted and the MW of the extracted materials is clearly shown. Median $\overline{M}w$ and $\overline{M}n$ values of the low density polyethylene hexane extracts were 1808 and 936, respectively, but were only 312 and 289, respectively, for the high density extracts. A plot of weight percent vs MW for the hexane extractables from a low density and a high density resin is shown in Figure 1; the samples were selected as representative of these 2 density groups and indicated the MWD of these low MW hexane extractable materials.

Effects of Chain Branching

The MW range of the material extracted by hexane from the high density resins was about 100–500, and that from the low density resins was about 100–5000, a much broader range (Figure 1). Chemically, the only difference between low and high density polyethylene is that long and short chain branches are present only in low density resins. This large difference in the observed MW ranges in the extracted material must therefore in some way be related to the presence of these branches. Conceivably, the presence of short chain branches in the oligomers of the low density polyethylenes enhanced their solubility in hexane (50°C) and enabled their extraction. However, numerous other factors such as solvent penetration effects, diffusion kinetics, and effective size must also be considered, and a complete explanation as to

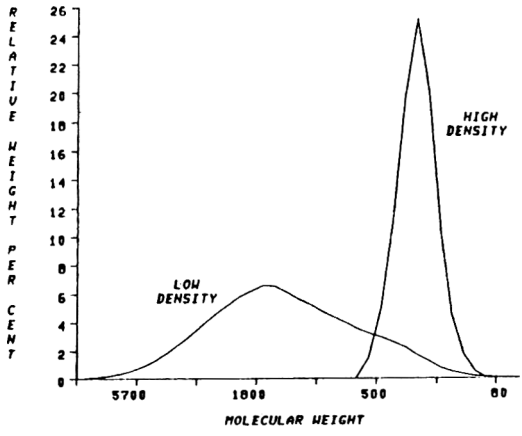

Figure 1. Hexane extractables from a high density (Sample 9) and a low density (Sample 6) polyethylene resin.

Table 4. Molecular weight distribution data for polyethylene xylene extractables

Sample	Wt % extractable[a]	$\overline{M}w$	$\overline{M}n$	Dispersity ($\overline{M}w/\overline{M}n$)
		Low Density Resins		
1	6.0	3195	1503	2.1
2	2.7	1810	1056	1.7
3	1.1	1831	1056	1.7
4	5.7	2366	103	22.9
5	3.9	2110	1284	1.6
6	2.9	2521	1494	1.7
Median	3.4	2305	1170	
		High Density Resins		
7	1.1	598	498	1.2
8	0.4	695	556	1.3
9	2.4	407	370	1.1
10	1.1	634	484	1.3
11	0.8	556	443	1.3
12	0.7	397	359	1.1
Median	1.1	577	464	

[a] Determined after xylene extraction procedure. Allowable limit 11.3%.

why such a large difference in MW range occurred goes beyond the scope of this work.

In spite of this large difference in the ranges of MW, the average amount of the very low MW oligomers (<500) extracted was essentially the same, regardless of the resin's density. The difference was oligomers of MW greater than 500 which could only be extracted from low density polyethylene. These higher MW oligomers extractable from the low density resins accounted for the larger extractable weight percent.

Xylene Extractable Material

The xylene extractable fractions of all 12 polyethylene resins, when characterized by SEC, showed the same general relationships (Table 4) as those observed for the hexane extractables (Table 3). The only significant difference was that xylene extracted a slightly higher MW fraction from each resin than did hexane (Figure 2). This increase was evidenced by both higher percent extractables and greater calculated $\overline{M}w$ values (Table 4).

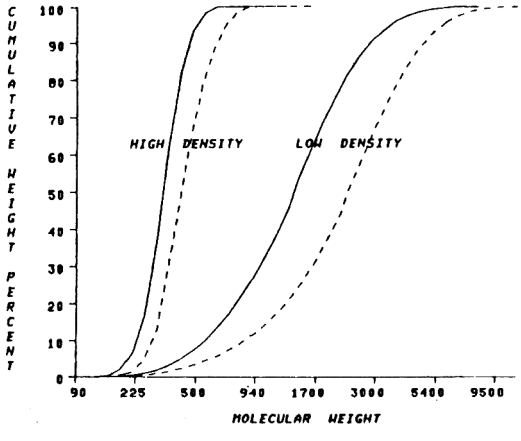

Figure 2. Hexane (solid line) and xylene (dashed line) extractables from a high density (Sample 9) and a low density (Sample 6) polyethylene resin.

Conclusion

The MWD of 12 food grade polyethylenes and their extractable oligomeric fractions have been characterized by SEC. Examination of these data revealed that the range of MW of the extractables depended on the resin's density; this dependence was considered to be due to the presence of short chain branching in low density polyethylene. Extractables from the low density resins had a much broader range of MW (about 100–5000) than did extractables from the unbranched, high density polyethylenes (about 100–500). A corresponding increase in the weight percent extractable from low density (about 3%) relative to the high density (about 0.5%) resins was also observed.

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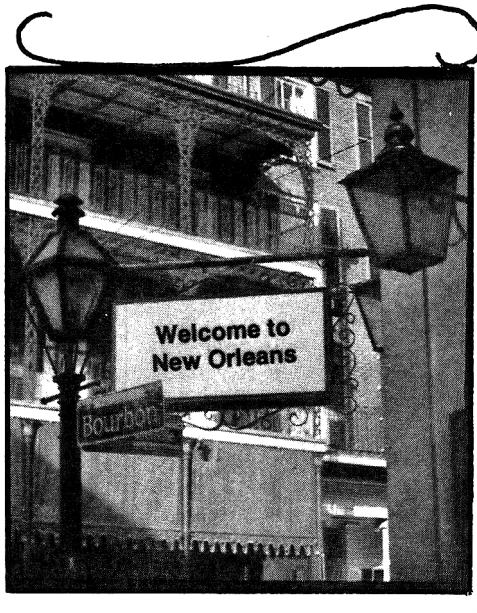

AOAC

METALS AND OTHER ELEMENTS

Lead in Preserved Duck Eggs: Field Screening Test and Confirmation and Quantitation by Atomic Absorption Spectrophotometry and Anodic Stripping Voltammetry

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Analysis of preserved duck eggs in this laboratory by acid digestion and atomic absorption spectrophotometry has shown that addition of lead salts to the egg coating to speed the preservation process results in lead in the egg albumen and yolk. The lead levels decrease as the lead passes through successive membranes, i.e., the lead level in the yolk is lower than that in the albumen. A method has been developed for the field screening of preserved duck eggs for the presence of lead in which a portion of the egg coating is shaken with 4% acetic acid, and the resulting solution is tested by a modification of the AOAC dithizone method. A quantitative method for laboratory confirmation of lead in egg albumen and/or yolk has also been developed in which lead is determined by atomic absorption spectrophotometry or anodic stripping voltammetry after closed-system Teflon-cup digestion with nitric acid.

During the past year and a half numerous samples of preserved and salted duck eggs have entered the Port of New York, from China, Taiwan, Hong Kong, and Thailand. The preserved duck eggs have an exterior pink, black, and/or brown mud-like coating that adheres to the shell. The edible portion consists of a blue-green to brown jelled albumen with a blue-gray semisoft yolk. The salted eggs have a black covering, and the edible portion consists of a slightly viscous yellow albumen with a darker yellow semisolid yolk.

These eggs were traditionally referred to as "thousand-year-old" eggs, because their preservation took a long time. Modern entrepreneurs speed up the "aging" process of these eggs by adding lead salts to the eggshell coating. The lead, on entering the egg, combines with sulfur to form colored gels or semisolid sulfides that give the aged appearance.

The Chinese Nationalist Government in Taiwan promulgated a National Standard for Alkalized Duck Eggs (1) as a guide for manufacturers. In addition, it set a Standard Method of Test for Alkalized Duck Eggs (2) which specified methods of testing materials used in the preparation of the eggs and methods for evaluating quality. Because the government was aware that lead was being added to the soaking solution and/or egg coating, the official standard prohibited the use of lead salts in the process. However, prohibition of lead did not prevent its use. The presence of lead in these imported eggs is the result of the intentional use of a non-permitted additive.

A rapid screening method has therefore been developed for detecting lead in preserved duck eggs based on acid extraction followed by a modification of the AOAC dithizone method, **25.035** and **25.037** (3). Results are confirmed and quantitated by a method for lead using anodic stripping voltammetry and atomic absorption spectrophotometry. Both methods are presented in this paper.

Lead Screening Test

The test is a modified version of the rapid screening method for cadmium and lead in earthenware, **25.035** and **25.037** (3, 4). In addition to the reagents listed in **25.035**, the following should be added to (i): "Add 20 mL 4% acetic acid to 8 dram screw-cap vial for field test (label this *leach solution*)".

To conduct the test, select 2 duck eggs at random from sample lot. Scrape coating with a Spoonulet (Fisher Scientific Co., Springfield, NJ 07081, Cat. No. 14-375-20 or equivalent); one filling of the Spoonulet will provide the approximately 1 g needed for test. Add material to vial containing leach solution, close vial, and shake ca 30 s; then let stand 2 min, and continue as in **25.037(a)**. A pinkish to red color in the $CHCl_3$ layer indicates a positive test for lead; a green to colorless layer indicates a negative test. If the test is positive for lead in the coating, lead will also be found in the albumen and yolk.

Samples giving positive results for lead can be

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confirmed and quantitated in the laboratory by anodic stripping voltammetry or atomic absorption spectrophotometry after closed-system Teflon®-cup digestion with nitric acid (5).

Quantitation of Lead

(Section numbers containing the letter A refer to the 1st supplement (6) to *Official Methods of Analysis*, 13th Ed.)

Apparatus

(a) *Polarograph.*—See **25.A01.**

(b) *Atomic absorption spectrophotometer.*—See **25.031.**

(c) *Decomposition vessel.*—70 mL. See **25.115.**

Reagents

(a) *Acids.*—(*1*) *Nitric acid.*—Redistilled. (*2*) *Perchloric acid.*—**See 25.A02(a)(*2*).**

(b) *Nitrate solution.*—See **25.A02(b).**

(c) *Lead standard solutions for anodic stripping voltammetry.*—See **25.A02(h).**

(d) *Lead standard solutions for atomic absorption spectrophotometry.*—(*1*) Stock solution.—See **25.A02(h)(*1*).** (*2*) Working solutions.—1.0, 2.0, 5.0, and 8.0 μg/mL. Pipet 1.0 mL stock solution into 100 mL volumetric flask and dilute to volume with 1% $HClO_4$. Pipet 1.0, 2.0, 5.0, and 8.0 mL diluted solution into separate 10 mL volumetric flasks and dilute to volume with 1% $HClO_4$.

Closed-System Digestion

Digest 1 g sample (wet weight) of material to be analyzed (coating composited by manual mixing; albumen, yolk, albumen-yolk composited in Waring blender) as in ref. 6. (Note: 1.0 g of each type of material to be analyzed was first tested to see if the addition of HNO_3 and heating on a hot plate produced a vigorous reaction. None was observed, and the closed-system digestion was presumed safe.)

Determination

Anodic stripping voltammetry.—Proceed as in **25.A04,** using 2 mL aliquot of digested sample for analysis.

Atomic absorption spectrophotometry.—Proceed as in **25.A05(c),** using 4.0 mL aliquot of digested sample and 0.4 mL 1% $HClO_4$. Dissolve residue in 1.0 mL 1% $HClO_4$. Operate instrument in accordance with manufacturer's instructions, using air-C_2H_2 flame and deuterium arc background corrector. Measure absorbance of sample and standards, (**d**)(2). Dilute sample solution with 1% $HClO_4$, if solution is too concentrated. Determine absorbance of sample and Pb working standard solutions. Flush burner with water and check zero point between readings. Determine Pb from standard curve of absorbance against μg Pb/mL or calibrate DCR unit in concentration mode with Pb working solutions, and read and record sample concentration directly. Bracket sample solutions with next higher and lower working solutions.

Table 1. Lead (ppm) found in preserved duck eggs (wet weight basis)

Sample No.	Field tests	Level		
		Coating	Albumen	Yolk
1–5	+[a]	1168–1375	2.3–8.5	1.5–4.0
6–7	+	1714–1800	3.8–5.0	1.5–2.0
8	+	1825	10.8	3.5
9	+	2574	27.0	5.9
10	+	3583	80.0	8.5

[a] + indicates a positive test.

Results and Discussion

We have found lead in almost all samples of preserved duck eggs entering the country through the Port of New York. Coatings to which lead has been added contained in excess of 300 ppm, and both the albumen and yolk contained lead (the lead level decreases as it moves through successive membranes) (Table 1). Subsequent samples containing high levels of lead in the coating were analyzed by compositing the albumen and yolk in a Waring blender. The results of these analyses are shown in Table 2. In all cases where coatings contained little (less than 20 ppm) or no lead, none was detected in the egg.

The rapid test for lead, described above, which takes 5 min for screening preserved duck eggs, was developed to expedite the analysis of these

Table 2. Lead (ppm) found in composited samples of preserved duck eggs (wet weight basis)

Sample No.	Field tests	Level	
		Coating	Composite of albumen and yolk
11–13	+[a]	380– 956	6.2– 8.9
14–16	+	1127–1474	5.5– 8.3
17–18	+	1797–1900	12.9–17.8
19–21	+	2375–2670	25.5–30.7

[a] + indicates a positive test.

Table 3. Lead (ppm) by atomic absorption spectrophotometry (AAS) and anodic stripping voltammetry (ASV)

Sample No.	AAS	ASV
22	6.0	5.8
23	7.3	7.4
24	21.2	21.1
25	1.9	1.8
26	3.1	2.9

samples. This study has shown that the screening method is reliable in determining whether egg albumen and yolk will contain lead. The sensitivity of the test has been adjusted so that low levels of lead (0–20 ppm) will give a negative test. Though the test will detect as little as 0.3 ppm lead/mL 4% acetic acid, the 30 s extraction of a 20 ppm coating, under the conditions of this test, will give a negative result. Lead in the coating at these low levels can be assumed to be a contaminant which will not migrate into the egg. When lead has been added intentionally, the levels are high and lead is found internally. Table 3 shows that both anodic stripping voltammetry and atomic absorption spectrophotometry give comparable results when these techniques are used to quantitate lead in duck egg samples.

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Thin Layer Chromatographic and Atomic Absorption Spectrophotometric Determination of Methyl Mercury

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A method is presented for determining methyl mercury that combines thin layer chromatography and atomic absorption spectrophotometry. The procedure is based on a previously reported method and entails isolating methyl mercury on a thin layer chromatogram, scraping adsorbent into an ignition tube, heating the tube, and moving the mercury vapor under vacuum into an absorption cell where it absorbs light at 253.7 nm. The procedure is sensitive to 0.7 ng Hg. Methyl mercury is extracted from biological samples by distilling, and then extracting with dithizone-chloroform. The extract is concentrated and applied to a thin layer chromatographic plate. The overall sensitivity of the method varies with sample size. For 100 g samples, the method is sensitive to about 9 ng Hg (as methyl mercury)/g (9 ppb).

Methyl mercury is toxic to humans and other animals. It occurs in the environment as a result of transformations of other forms of mercury, and tends to accumulate in organisms. Thus quantitative analytical methods for determining methyl mercury should be widely available for medical and environmental studies of this compound.

Methyl mercury has been determined almost exclusively by gas-liquid chromatography with electron capture detection. Thin layer chromatography (TLC) and atomic absorption spectrophotometry (AAS) have also been used for methyl mercury determinations. However, their combined use has received little attention. The method reported here is simpler and more sensitive than previous methods, such as that of Yamaguchi et al. (1).

To apply the assay presented in this paper, an extraction procedure for methyl mercury was needed. Extraction procedures reported for methyl mercury are suitable for analysis by gas chromatography, but not by AAS unless the samples contain a relatively large amount of methyl mercury (e.g., 0.3 μg Hg as methyl mercury/g). This is because the more sensitive electron capture gas chromatographic methods can tolerate large losses of methyl mercury resulting from unfavorable partitioning and solvent losses during extraction. Distillation of the methyl mercury, as reported by Yamaguchi et al. (1), would allow the use of larger samples and would eliminate large solvent losses in the sample; a single-solvent extraction of the distillate would minimize partitioning losses, compensating for the lower sensitivity of AAS. Some modifications are described below.

Experimental

Extraction of Methyl Mercury

Distillations were performed using an all-glass still with a 50 cm long West-type condenser cooled with ice water circulating between the condenser and a bucket via an electric pump. A few glass beads (0.3 mm diameter) were included in the retort (2 L round-bottom boiling flask) to prevent bumping. The sample was boiled with 50 mL 12 N HCl, 50 mL 1M $CuSO_4 \cdot 5H_2O$ (to mask sulfhydryl groups (2)), and enough purified water to make ca 1 L total volume, until 500 mL distillate was collected. After distillation, the condenser, the connector to the collecting vessel, and the collecting vessel (graduated Erlenmeyer flask) were rinsed with purified (distilled, deionized) water, and the rinse was added to the distillate.

The distillate was extracted twice with 10 mL dithizone-chloroform (0.0006%) and once with 5 mL chloroform. The extract was dehydrated in a 15 mL plastic conical centrifuge tube with a hole bored in its bottom. A small plug of glass wool was placed in the bottom, and about 1 g granular anhydrous sodium sulfate was poured on top of the glass wool. A rubber stopper (No. 00) was pierced with a hypodermic needle that was attached to a rubber hose. Air pressure was applied to the hose so that the extract could be gently forced from the centrifuge tube into a graduated cylinder by placing the stopper into the tube. The extract (of known volume) was then concentrated to 1.0 mL in a conical 15 mL graduated centrifuge tube in a 50°C water bath with the aid of a vacuum hose.

This extraction procedure from purified water gave 100% recovery of methyl mercury. During solvent extraction, only about 70% of the solvent was recovered because it had a tendency to stick

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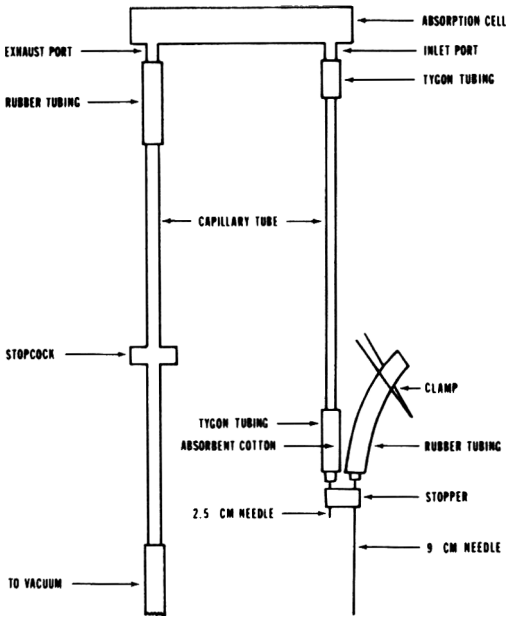

Figure 1. Schematic diagram of mercury pyrolysis and measurement apparatus (not to scale).

Operation: connect ignition tube to stopper; create vacuum in system; heat ignition tube; open clamp to obtain absorption peak; evacuate system.

to the walls of the separatory funnel. This loss was not reduced by cleansing with detergent, chromic acid, 1N ammonium hydroxide, ethanol, or acetone.

Total Mercury Determination

To choose proper sample sizes for methyl mercury analysis, total mercury in the samples was determined by digesting the sample, reducing mercury to elemental mercury, and aerating and trapping the elemental mercury in a permanganate solution (3), which was then extracted twice with 5 mL dithizone–chloroform (0.0006%) and once with 3 mL chloroform (4).

Mercury standards were prepared by extracting a mercuric chloride solution with dithizone–chloroform (4). Sample extracts and standards were pipetted into ignition tubes in appropriate volumes (not exceeding 1 mL), evaporated to dryness in a 50°C water bath under a stream of nitrogen gas, and analyzed for mercury, as described for methyl mercury below, by atomic absorption spectrophotometry.

Thin Layer Chromatography

Thin layer chromatographic plates were prepared using the Desaga–Brinkmann spreading apparatus (Westbury, NY) to coat 20 cm square glass plates with 0.25 mm thick silica gel 7 without binder (J.T. Baker Chemical Co., Phillipsburg, NJ). Immediately before use, coated plates were activated ca 3 h at 150°C.

The concentrated extract was spotted in duplicate 2.0 and 4.0 μL volumes for ascending TLC. A methylmercuric dithizonate standard was prepared to contain ca 10 ± 0.1 μg Hg (as methylmercuric chloride) and 0.1 mg dithizone/mL benzene. Using a 5 μL syringe, the standard was spotted in duplicate 0.5, 1.0, and 2.0 μL volumes, giving 5, 10, and 20 ng Hg, respectively, per spot. A reference spot containing 0.2 μg Hg (as methylmercuric dithizonate) was added for locating standards and unknowns after development. The developing solvent (5), petroleum ether–acetone (9 + 1), was allowed to migrate 10 cm. After development, a 4 cm wide silica gel band (2 cm above and below center of reference spot) containing methyl mercury was scraped onto weighing paper and placed in an ignition tube for mercury determination by AAS. Retention factors for methylmercuric dithizonate and mercuric dithizonate were 0.83 and 0.43, respectively.

Atomic Absorption Spectrophotometry

Atomic absorption spectrophotometry was carried out with a Perkin-Elmer Model 303 atomic absorption spectrophotometer equipped with an automatic chart recorder (Hewlett Packard). The absorption cell (Figure 1) was constructed according to Hatch and Ott (6) (15 × 2.5 cm od borosilicate glass tubing with quartz windows cemented on with G.E. Silicone Seal). Glass inlet and outlet ports were blown on near each end of the absorption cell, which was mounted to the nebulizer of the spectrophotometer through a series of clamps, allowing its easy alignment in the light path.

To the inlet port of the absorption cell was attached (via a Tygon tubing joint) a 23 cm length of glass capillary tubing (approximately 1.8 mm id) bearing a Tygon tube containing a 2.5 cm hypodermic needle inserted through a rubber septum from a Vacutainer (Becton, Dickinson & Co., Rutherford, NJ). A longer (9 cm) needle was also inserted through the septum, and a short piece of rubber tubing was attached to it and clamped with a spring clamp. The ignition tubes (16 mm od × 125 mm; 915°C softening point, No. 1720 Pyrex glass; A. H. Thomas Co., Philadelphia, PA; No. 9225-L41) could be easily and snugly fitted to the stopper. A stopcock was attached by rubber tubing to the outlet port. The

Table 1. Determination of methyl mercury in fish and sewage sludge

Sample	Total Hg found[a]	Methyl mercury found (μg Hg)[a]		
		Before spiking	After spiking[b]	
			Hg added, μg	Rec., %
Tuna, 100 g	0.105	0.056	4.8	90
Trout,[c] 1.0 g	7.5	5.26	—	—
Sludge, 100 mL	0.130	0.012	9.6	73

[a] Concentrations in μg/g for fish and μg/mL for sewage.
[b] Methyl mercury was added as the dithizonate.
[c] From lake highly contaminated with mercury; substantially dehydrated when analyzed.

proximal end of the stopcock was replaced with capillary tubing to reduce its volume, and the distal end of the stopcock was attached by rubber tubing either to a vacuum pump (Millipore Corp., Bedford, MA) or to the laboratory vacuum line. The vacuum pump was used to create a vacuum in the system, and the laboratory vacuum line was used for evacuating the system at the end of a trial. The protocol for the spectrophotometric procedure follows.

Prepare spectrophotometer for operation according to manufacturer's instructions for determination of mercury, using scale expansion X1 or X3, noise suppression 2 or 3, and chart speed 0.25 in./min. Align absorption cell to permit maximum transmittance of incident light. Snugly fit ignition tube containing sample to be analyzed to rubber stopper, clamp off long needle, and connect stopcock to vacuum pump. Start pump and open stopcock until desired vacuum of 15 in. Hg is attained. Close stopcock and switch off pump. Heat bottom end of ignition tube with Meker-type (Fisher) burner (held by hand) for 20 s. Open clamp. Following occurrence and stabilization (ca 2–3 s) of absorption peak, flush system with laboratory vacuum. Absorption is proportional to peak height.

Results

Variation in absorption peaks of replicate samples is reasonably low. For example, 5 replicate samples of 0.2 μg Hg (as mercuric chloride) giving absorption peaks about 55% had a 3% coefficient of variation [(standard deviation/mean) × 100]. The relationship between absorbance and mercury concentration was linear. The sensitivity was approximately 0.7 ng Hg, when the blank gave negligible absorption.

The absorbent cotton in the absorption cell apparatus required replacement occasionally because of clogging with silica gel. This was indicated when the vacuum drop through the open system diminished. Also, the absorption cell was infrequently cleaned by flushing with ethanol.

Sample Analysis

Fish and anaerobically digested sewage sludge were analyzed for total mercury and methyl mercury, and recovery of added methyl mercury (dithizonate) was tested. Tuna was prepared by homogenizing 250 g canned tuna in a blender and diluting the homogenate to 1 L to give 0.25 g tuna/mL, ensuring uniform samples. A 10 g sample of trout was prepared in a similar manner. Sewage sludge samples were taken by pipet while mixing with a magnetic stirrer. Total mercury analyses showed 0.105 μg Hg/g in tuna, 7.5 μg Hg/g in trout, and 0.130 μg Hg/mL in sewage sludge. The mercury concentration in the trout was high because it was obtained from a lake highly contaminated with mercury and because it was substantially dehydrated when analyzed.

After total mercury analysis, sample sizes of 100 g tuna, 1 g trout, and 100 mL sewage sludge were chosen for methyl mercury analysis. The samples were extracted as described above. Methylmercuric dithizonate standards and sample extracts were spotted in duplicate on chromatographic plates and analyzed for mercury by AAS. The methyl mercury contents of the tuna, trout, and sewage sludge were 0.056 μg/g, 5.26 μg/g, and 0.012 μg/mL, respectively.

To test the recovery of methyl mercury, 4.8 and 9.6 μg Hg (as methyl mercury) were added to, respectively, 100 g tuna and 100 mL sewage sludge, previously analyzed. The samples were then re-analyzed for methyl mercury. Ninety percent of the added methyl mercury was recovered from tuna, and 73% from sewage sludge (Table 1). This indicated adequate recovery for most methyl mercury analyses. These results cannot be compared with those of Yamaguchi et al. (1) because the latter reported no performance

data. Westöö (7) reported a mean recovery of 90% (SD 6%) from fish and other foods, and Uthe et al. (2) reported a recovery of 99% (SD 5%) from fish for analysis by gas chromatography.

Discussion

The AAS method presented here can be used for both methyl mercury (in conjunction with TLC) and total mercury analysis using equipment commonly available in water quality laboratories. Mercury vapor meters have been used in similar methods (4, 8) instead of a costlier commercial atomic absorption spectrophotometer.

The sensitivity of the present method compares favorably with other atomic absorption procedures. Yamaguchi and Matsumoto (8) reported 7 ng Hg as the lower end of the "measurable range," and Rathje (9) reported that 6 ng Hg could be "detected with no difficulty." Rathje's method was similar to that of Hatch and Ott (6) except that it was an open system rather than a closed one. Reporting on the further development of the syringe technique, Deitz et al. (10) claimed a 2 ng level of "determination." The sensitivity of the present method was typically 2.3 ng Hg in the methyl mercury determination (silica gel blank gave approximately 1% absorption) and 0.7 ng in the total mercury determination where the absorption of the dithizone–chloroform blank was nil.

Methyl mercury determination in fish samples by gas chromatography is sensitive to 0.01 μg methyl mercury/g (2, 11). The present method is sensitive to about 0.009 μg Hg/g when 100 g samples are analyzed, indicating sensitivity to be of practical value.

In conclusion, the method for methyl mercury analysis presented in this paper is a practicable alternative to gas chromatography. Although only fish and sewage sludge were analyzed by the present method, a large variety of samples (e.g., foods, sediments, soils, effluents) could likely be successfully analyzed for methyl mercury. Encouraging preliminary results were obtained on sediment. Several cold-vapor methods for determining mercury by AAS have been published. This paper presents yet another variation that, in contrast to most other analytical methods in common use, can be used for both organic mercury (in conjunction with TLC) and total mercury analysis.

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Microdiffusion and Fluoride-Specific Electrode Determination of Fluoride in Infant Foods: Collaborative Study

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Twelve laboratories analyzed (1 replicate) 12 samples of infant foods – milk, pears, and peas – containing 0.2-5 ppm F. There was one laboratory outlier. Mean coefficients of variation were 7.06% for intralaboratory determination of 3 sets of blind duplicates and 21.6% for interlaboratory determination of 12 samples. Variance analysis for all samples yielded a reproducibility standard deviation of 0.41 ppm; for 3 sets of blind duplicates, repeatability standard deviation was 0.26 ppm and reproducibility standard deviation was 0.32 ppm.

Current AOAC methods for determining fluoride (F) lack the sensitivity for accurately determining low levels of F in foods. Problems are caused by high blanks (1) and the slowness and questionable accuracy of direct standard addition methods using F-specific electrodes (2, 3). The reliability of colorimetric procedures, even after separation of F from the bulk of the matrix, has been questioned (4, 5). Occasionally, foods require ashing (1).

A recently published method (6) for determining F in unashed foods, using microdiffusion from 40% perchloric acid and determination by the F-specific electrode, appeared more promising than the aforementioned methods with respect to sensitivity, simplicity, speed, and accuracy; therefore, a collaborative study was undertaken to evaluate the performance of the method with infant foods in different laboratories.

Collaborative Study

Preparation of samples.—A single lot of each of 3 foods—strained peas, strained pears, and evaporated whole milk—was purchased commercially. Each food was blended and divided into 3 portions, and 2 of the portions were spiked with F at 2 levels. Each portion was then blended, freeze-dried, ground to a powder, and mixed.

Samples, 10 replicates of each, were tested for F homogeneity by using the published method (6); homogeneity for all samples was within 5% of the mean value. Samples were then transferred to polyethylene bottles and 5 mL polystyrene vials.

Design of study.—Each collaborator was sent 12 vials, comprised of 4 milk, 4 pea, and 4 pear samples. Two of each 4 samples were blind duplicates. The choice of samples and spiking levels enabled evaluation of the method at 3 fluoride levels: 0.2–1.0 μg/g, 1.4–2.0 μg/g, and 3.5–5.0 μg/g. A blind duplicate was included for each of these ranges.

Collaborative kits.—Each collaborator was sent a kit containing: 31 Petri dishes (Millipore Corp., Cat. No. 10-040-00), one of which was spotted with 0.1 mL 0.5M NaOH; ca 9.5 g Ag_2SO_4; 80 mL commercial TISAB-II (Orion No. 94-09-09A); sodium fluoride standards containing 100 and 10 ppm F; a vial of practice sample (NBS Orchard Leaves, SRM 1571); 12 vials of unknown samples; instructions; graph paper; and a sample calibration curve.

Instructions.—Collaborators were instructed to analyze 2 replicates of the practice sample (NBS Orchard Leaves, SRM 1571); an acceptable F level for this sample was 3–5 μg/g. Upon satisfactory completion of this step, each of the 12 unknowns was to be analyzed once. Instructions for the collaborative study essentially followed the method described by Dabeka et al. (6) with the following additions and modifications:

General precautions: Concentrations of F used in study vary by 3–4 orders of magnitude, and risk of cross contamination is large for personnel unacquainted with trace and ultra trace analysis. Guard against contamination from tap water and fingers.

Diffused standards: Use 4 standards, containing 0.1, 0.3, 1.0, and 10.0 μg F, for practice sample; use 6 standards (0.01, 0.03, 0.1, 0.3, 1.0, and 10.0 μg F) for unknowns. Use calibrated (0.1 mL) pipets.

Spotting and drying: For even distribution of spots, hold finger on upper aperture of 0.1 mL

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pipet while touching tip to lid. After spotting, snugly cover lid with base of Petri dish, keeping dishes horizontal. Dishes must be covered to avoid absorption of carbon dioxide from the air. Oven-dry at 60°C for 24–48 h.

Diffusion conditions: Diffuse samples at 47 ± 2°C for 16 h. Petri dishes should not be stacked during this step, to avoid condensation of water on lid.

Measurements: Soak electrodes in beaker of water ≥24 h before use. Electrodes thus immersed usually require conditioning for accurate measurement of low fluoride concentrations, which is done immediately before determining standards and samples by measuring a standard fluoride solution containing ca 5 ppm fluoride in 50% TISAB-II until mV readings stabilize. Then, electrodes are immersed 60 s in water, with measurement mode on. In practice, standard containing highest level of fluoride is measured first. Order of measurement for other standards and samples is irrelevant; however, all should be measured the same day. All mV readings should be allowed to stabilize (5–30 min) until changes in either direction do not exceed 0.2 mV/min. Record readings to nearest 0.1 mV. Between all sample and/or standard readings, rinse electrodes in 250 mL beaker containing distilled water, avoiding physical contact of beaker, etc., with F-specific electrode. With electrodes immersed in rinse water, set pH/mV meter to measurement mode for 60 s to release residual fluoride. Gently dab dry electrodes with clean cellulose wipes. Rinse interior and exterior of measuring dish with deionized water and dry with clean cellulose wipe.

Results and Discussion

Twelve collaborators participated in the study, and results were reported over a 9-month period (July 1979–March 1980). Collaborators reported general problems and comments as follows: The pear samples were hygroscopic, difficult to weigh, and appeared to contain a volatile constituent which was absorbed by the NaOH on the lid. It was difficult to wet some of the samples when $HClO_4$ was added. After diffusion, it was difficult to dissolve the NaOH spots in TISAB-II (1 + 1) because of the affinity of the solvent for the edge of the Petri dish. The way TISAB-II solution adhered to the lids after diffusion was different for the 3 foods. More standards should have been included.

Specific problems encountered by collaborators were: Laboratory 6 lost the 0.1 μg (1 μg/g) standard when diffusing the unknowns. Laboratory 1 incorrectly drew the calibration curve and incorrectly calculated the concentration for some samples. The results for Laboratory 12 indicated a possible contamination problem. Millivolt readings for the 0.01, 0.03, and 0.1 μg F standards corresponded with F levels of approximately 1.5, 0.67, and 4.5 μg F, respectively. During analysis of the practice sample, however, no problem was encountered. Laboratories 1 and 4 weighed samples only within 0.01 g, an oversight in instruction clarity. Laboratory 8 recorded readings to ±1 mV instead of ±0.1 mV.

The submitted results were corrected for errors by redrawing the calibration curve (Laboratory 1), were rounded to 2 figures, and evaluated statistically according to Youden and Steiner (7). Results reported by Laboratory 12 were excluded from the data because problems were encountered with the sample calibration curve, results ranged from 1.2 to 68 ppm for the samples, and ranking the results indicated that the laboratory was an outlier. The corrected results from all other laboratories are presented in Table 1.

For the remaining data (Table 1), Dixon's test revealed that 6 individual values were outliers. These were from Laboratory 1 (Sample 9), Laboratory 2 (Sample 4), Laboratory 4 (Samples 1, 2, and 3), and Laboratory 8 (Sample 4). These outliers appeared to be random, with the exception of those of Laboratory 4. Division of the 12 samples into 3 categories according to F level, followed by ranking the values by laboratory, revealed that Laboratory 4 was an outlier for the samples with low F levels (Samples 1, 2, 5, and 9).

Homogeneity of variation between laboratories was only approximate (0.17 calculated versus 0.15 theoretical at the 95% confidence level). Variation between replicates was not homogeneous (0.20 versus 0.12), primarily because the logarithmic relationship between potential and concentration results in standard deviations which are approximately proportional to concentration on the linear portion of the calibration curve. Nonetheless, statistical analysis of all the data in Table 1 yielded an overall reproducibility standard deviation of 0.41 ppm (Table 2).

For the 3 sets of blind duplicates, the reproducibility standard deviation was 0.32 μg/g, and the repeatability standard deviation was 0.26 μg/g. The true variance between laboratories ($\sigma_L^2 = -0.0018$) and the true variance of the laboratory–sample interaction ($\sigma_{LS}^2 = 0.033$) were small, and variance ratios ($MS_L/MS_{LS} = 0.92 < 2.8$ and $MS_{LS}/MS_0 = 1.96 < 2.1$) were not significant,

Table 1. Refined[a] collaborative results (μg/g) for F in infant foods

Lab.	1	2	3	4	5	6	7	8	9	10	11	12	Practice	
1[b]	0.76	0.87	2.2	4.4	0.66	1.7	3.4	3.7	2.1	2.2	2.0	4.3	2.8[c]	2.2[c]
2	0.79	0.85	2.3	5.3	0.63	1.4	4.4	4.8	0.29	1.7	2.5	5.7	4.1	3.9
3	1.1	0.94	2.1	4.3	0.88	1.2	3.7	4.0	0.48	1.8	2.2	5.5	3.7	3.9
4	1.5	1.5	3.0	4.5	1.1	1.9	4.5	3.0	1.0	1.9	2.0	3.1	3.3	3.2
5	0.89	0.96	2.1	4.4	0.69	1.6	4.3	4.5	0.35	2.2	2.3	5.8	3.6	3.7
6	0.62	0.78	1.8	4.1	0.81	1.6	4.0	4.3	0.23	2.4	2.1	5.4	3.6	3.8
7	1.1	1.2	1.8	3.8	0.76	1.4	3.7	3.6	0.42	1.9	2.0	5.2	3.8	3.5
8	0.84	0.90	1.6	3.4	0.81	1.6	3.2	4.0	0.80	1.9	2.2	4.4	3.3	3.5
9	0.89	0.81	1.6	4.2	0.62	1.4	3.6	3.7	0.35	1.7	1.7	5.1	3.6	3.6
10	0.91	0.96	1.8	4.5	0.77	1.6	3.8	3.9	0.42	2.1	2.0	5.5	3.5	3.8
11	0.75	0.83	1.5	4.2	0.89	1.3	4.1	4.1	0.37	1.8	1.8	4.9	3.1	3.1
$\bar{x}$	0.92	0.96	1.98	4.28	0.78	1.52	3.88	3.96	0.62	1.96	2.07	4.99		
SD	0.24	0.21	0.43	0.47	0.14	0.20	0.42	0.48	0.54	0.23	0.22	0.79		
RSD, %	25.89	21.89	21.62	10.99	17.92	13.11	10.73	12.17	87.52	11.67	10.81	15.91		

[a] Rounded to 2 significant figures.
[b] Corrected for errors due to calculations and calibration curve.
[c] Practice sample values fell outside acceptable limits and the fault was traced to a high oven temperature. Laboratory 1 was instructed to continue the study after the corrections of the problem.

indicating that the method is satisfactory.

Relative standard deviations (RSDs) for the 12 samples varied between 10.8 and 87.5% (Table 1) with a mean of 21.68%. Rejection of individual outliers reduces the mean RSD to 15.2%.

Including all results, the mean sample RSDs, divided according to F level, decrease with increasing F level (Table 2). The effect of food product on RSD is somewhat ambiguous (Table 2) because of the high RSD for Sample 9 (pears).

RSDs between blind duplicates varied, overall, between 0.0 and 28.2%, with a mean value of 7.06%. Differences among the 3 F levels (and among the 3 food products) were not significant. For milk, peas, and pears, the mean RSDs between duplicates were 6.90, 6.88, and 7.40%, respectively.

Table 2. Statistical analysis on all results from Laboratories 1–11

True variance of the laboratory-sample interaction,[a] σLS^2	0.0330
True variance between laboratories,[a] σL^2	−0.00180
Repeatability standard deviation[a] (ppm)	0.262
Reproducibility standard deviation[a] (ppm)	0.316

Fluoride range	Food product	Reprod. SD, ppm	Mean sample RSDs, %
All	all	0.408	21.7
0.2–1.0	all	0.322	38.3
1.4–2.0	all	0.285	14.3
3.8–5.0	all	0.561	12.5
All	milk	0.356	18.8[b]
All	peas	0.341	14.2[b]
All	pears	0.507	37.5[b]

[a] For 3 sets of blind duplicates.
[b] Calculations based on mean of 3 values, one of which is average RSD for the blind duplicates.

While the above data demonstrate that the method performed reasonably well for 11 of the 12 participating laboratories, the data include (a) 6 individual outliers, (b) 1 laboratory outlier with respect to the low-level samples (Laboratory 4), (c) 1 laboratory (8) which recorded readings only to the nearest 1 mV, and (d) 2 laboratories (1 and 4) that weighed samples to the nearest 0.01 g. Rejection of the results from the above 3 laboratories (1, 4 and 8), primarily to maintain a symmetrical matrix, simultaneously eliminates all but 1 individual outlier (Sample 4, Laboratory 2) and reduces the overall reproducibility standard deviation from 0.41 to 0.24 ppm. Furthermore, for the samples with low, intermediate, and high F levels, the reproducibility standard deviations, 0.13, 0.24, and 0.31 ppm, respectively, increase with increasing F level. The mean for Sample 9 is reduced from 0.62 ppm to 0.36 ± 0.08 ppm, a value which more closely agrees with that (0.27 ± 0.06 ppm) found by the authors. While the above manipulation is not representative of the collaborative results as a whole, it does indicate that 3 laboratories may have had more problems with the method, and that 8 of the 12 participating laboratories were able to obtain results for all samples which were in excellent agreement.

The method, therefore, appears satisfactory, and the good intralaboratory precision for blind duplicates (7.06% mean RSD) indicates that there are relatively few procedural problems.

Table 3. Fluoride levels in collaborative samples before and after freeze-drying

Food	Sample No.	Mean F level, Coll. 1–11 (μg/g ± SD)	F found, this lab. (μg/g, 99% CI)	F added before freeze-drying (μg/g, dry wt)	% Recovery based on: Results of coll.	% Recovery based on: Results of this lab.
Milk A	1	0.92 ± 0.24	0.80 ± 0.14	—		
Milk A	2	0.96 ± 0.21	0.80 ± 0.14	—		
Milk B	3	1.98 ± 0.43	1.72 ± 0.16	0.95	109	98
Milk C	4	4.28 ± 0.47	4.3 ± 0.4	3.8	88	93
Peas A	5	0.78 ± 0.14	0.61 ± 0.09	—		
Peas B	6	1.52 ± 0.20	1.42 ± 0.15	0.91	81	93
Peas C	7	3.88 ± 0.42	3.8 ± 0.3	3.6	86	90
Peas C	8	3.96 ± 0.48	3.8 ± 0.3	3.6	88	
Pears A	9	0.62 ± 0.54	0.27 ± 0.06	—		
Pears B	10	1.96 ± 0.23	1.9 ± 0.3	2.5	54	69
Pears B	11	2.07 ± 0.22	1.9 ± 0.3	2.5	58	
Pears C	12	4.99 ± 0.79	4.9 ± 0.6	6.7	65	70

Accuracy

Although the accuracy of the method was adequately tested before publication (6), mean recoveries of F added to samples before freeze-drying ranged from 54 to 109% by collaborators and from 69 to 98% by the authors (Table 3); therefore, it appears that some F is lost during freeze-drying. These losses are related to food type (Table 3) and are not universal (see Table 2, reference 6). However, the possibility of their occurrence suggests the elimination of the freeze-drying step in the analysis of foods. The method, as originally published (6), allows for such a modification.

Generally, the F levels obtained by collaborators agreed well with those found by the authors using different sample sizes, 3 sets of calibration curves per day, and 8 standard concentrations for each curve (Table 3). Nonetheless, 3 of the 11 acceptable laboratories obtained unusually high results for Sample 9, unspiked pears, and some problems can be anticipated when determining F at such low levels.

Considerations for Method Improvement

The intralaboratory precision obtained in this study for blind duplicates enables laboratories which want to further improve accuracy of results to do so via minor changes to the method.

Table 4 lists the reported mV readings for the 4 standards common to the calibration curves of the practice sample and the unknowns. Significant shifts (ΔmV) of the mV readings between the 2 days the practice sample and unknowns were analyzed occurred for Laboratories 1, 5, 6, 11, and 12, suggesting that electrode drift occurred. Drift can occur even over a period of several hours (8), and its effects on accuracy can be reduced by running a calibration curve both before and after analysis of samples.

Table 4 also illustrates that the above shifts of the mV readings (ΔmV) may change from stan-

Table 4. Millivolt readings reported by collaborators for 4 standards common to calibration curves of practice sample (A) and unknowns (B)

Lab.	0.1 μg F A	0.1 μg F B	0.1 μg F ΔmV	0.3 μg F A	0.3 μg F B	0.3 μg F ΔmV	1.0 μg F A	1.0 μg F B	1.0 μg F ΔmV	10.0 μg F A	10.0 μg F B	10.0 μg F ΔmV	$\frac{\Delta mV_{max}}{\Delta mV_{min}}$
1	361.8	404.0	42.2	328.9	379.4	50.5	301.0	344.9	43.9	264.8	302.2	37.4	13.1
2	151.5	149.8	−1.7	127.9	130.8	2.9	98.5	—	—	39.6	43.7	4.1	5.8
3	−119	−120	1	−143	−145	−2	−176	−176	0	−237	−235	2	4
4	−121.8	−109.5	−12.3	−142.8	−139.2	3.6	−173.3	−169.5	3.8	−230.7	−229.3	1.4	16.1
5	160.1	150.4	−9.7	136.5	126.1	−10.4	105.9	96.2	−9.7	48.8	38.2	−10.6	0.9
6	160.7	—	—	136.4	121.8	−14.6	106.7	92.8	−13.9	47.2	36.0	−11.2	3.4
7	183.2	187.1	3.9	160.2	154.9	−5.3	127.8	126.6	−1.2	71.8	77.4	5.6	10.9
8	154	158	4	135	137	2	109	113	4	54	58	4	2
9	13.5	14.4	0.9	−12.6	−12.1	0.5	−42.4	−41.8	0.6	−102.7	−100.8	1.9	1.1
10	166.4	167.2	0.8	140.7	140.4	−0.3	109.3	109.6	0.3	53.5	51.7	−1.8	2.6
11	213.4	241.7	28.3	188.7	219.6	30.9	159.4	192.6	33.2	103.9	132.0	28.1	5.1
12	141.0	38.9	−102.1	119.3	104.2	−15.1	97.2	79.8	−17.4	31.4	24.0	−7.4	94.7

dard to standard, and the maximum shifts ($mV_{max} - mV_{min}$) are listed in the last column. Large maximum shifts are evident for Laboratories 1, 2, 4, 7, 11, and 12, and are an indication of the reproducibility of the standard curve as represented by the individual standards. Increasing the number of standards, particularly in the low concentration region where the curve is nonlinear (0.5–0.01 μg F) and contamination problems are greatest, should also improve accuracy.

The third step in improving accuracy is to vary the sample size (between 0.05 and 0.2 g) to confirm (a) noninterference by other sample components which may be diffused, and (b) quantitative diffusion and absorption of HF. After the collaborative study was completed, a correlation was found for the pear samples between sample size and recovery. Sample sizes exceeding 0.1 g yielded poorer recovery. This was overcome by increasing the volume of perchloric acid added to the sample before diffusion, from 2 mL to 3 mL. Such a modification to the method did not affect results and enables analysis of up to 0.15 mg food, thus improving detection limits. Increased amounts of Ag_2SO_4 are not required for this modification. Further increasing the volume of perchloric acid to 5 mL is being investigated.

Fourth, collaborators were instructed to record mV readings when their drift was reduced to 0.2 mV/min. At low F concentrations, this state may not correspond with equilibrium conditions, and complete equilibration of mV readings may prove more satisfactory. This is important because, at low F concentrations, consecutive 2-directional drifts can occur before complete equilibration. A precaution for this approach, however, is that drainage of reference solution into the small volume of measured solution affects mV readings, and the equilibration time must be considered. Furthermore, we have observed that, at low F concentrations, the equilibrium state depends on whether the preceding solution measured contained a high level of F. This can be overcome by arranging the measurement sequence for samples and standards in order of decreasing concentration; however, this is only applicable when the approximate concentration of F in the sample is known, i.e., during analysis of reference materials. For samples with unknown F levels, it can be overcome by equilibrating the electrode between each measurement with water or TISAB-II containing no fluoride. This approach was taken in the collaborative study.

Finally, some modifications to technique which significantly shorten analytical time and facilitate manipulations are outlined below.

Spotting the lids with aqueous sodium hydroxide solution was tedious and, with time, the dried spots tended to flake and fall from the lid. These problems can be overcome by using an ethanol solution of NaOH, rapidly prepared in a 100 mL volumetric flask by dissolving 4 g sodium hydroxide in 10 mL water followed by dilution to volume with ethanol. Any precipitate (sodium carbonate) need not be filtered. Place the lids of the Petri dishes on a warm (100°C) hot plate and pipet 50 μL ethanolic NaOH onto each lid while touching the pipet tip to the lid and moving it in a spiral to distribute the solution. After partial drying, cover the lids with the bases and oven-dry 24 h at 60°C.

The problem of adherence of TISAB-II (1 + 1) to the edge of the lids after diffusion, making dissolution of spots difficult, can be overcome by pipetting the TISAB solution into the center of the lid and waiting 2–3 min until dissolution of the central position is complete. Subsequently, the solution is gently swirled to dissolve remaining portions on the lid.

The collaborative study has demonstrated that the method is precise and reproducible in different laboratories. Furthermore, the method is capable of accurate determination of background levels of F in foods and biological materials, and is flexible with respect to sample preparation (fresh or freeze-dried) and sample size (0.1 g for normal diffusion and 1.0 g for macrodiffusion) (6).

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Open Digestion Modification of AOAC Method for Determination of Mercury in Fish

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The vanadium pentoxide-catalyzed reflux method has been modified to allow open digestion of samples in preparation for determination of organic and inorganic mercury. The reflux digestion performed with a bunsen burner resulted in charred samples, but digestions performed in tubes in a 95-100°C water bath were satisfactory. As many as 40 digestions can be carried out simultaneously, and the technique offers a fast and simple approach to digestion, dilution, and transfer.

The original method of Hatch and Ott (1) for the determination of mercury in fish has seen several modifications over the years (2-5), particularly regarding the digestion of samples. In recent years, we have been responsible for carrying out mercury determinations on fish, and we use the vanadium pentoxide-catalyzed reflux digestion method of Munns and Holland (3, 6). Lately, we have been called on to analyze an increased number of fish samples for mercury content; therefore, we wished to modify the above method to handle more digestions per unit of time. Our modified open digestion technique is presented below.

Experimental

Method Techniques (3)

Carry out all digestions in hood. Use cold vapor technique given in references 3 and 6, except as follows: (a) Use open system, and use peak height rather than maximum absorbances for standard graph and calculation; (b) use 20 mL cut pipet containing blue coarse silica gel as moisture trap; and (c) after adding reducing solution, stir mixture vigorously 2½ min, and then purge with air.

Samples

Homogenize thoroughly fresh and canned tuna in Waring blender; use mortar and pestle to complete homogenization. Use standard powder without further preparation.

Procedure

Weigh 1.5-2.0 g prepared canned or fresh sample or 0.5-1.0 g standard powder into spouted 50 mL Nessleriser tube. Add 10-15 mg V_2O_5 and 6 mL HNO_3-H_2SO_4 (1 + 1) and leave at room

Table 1. Recoveries of added Hg (μg/g)

	Munns and Holland (3)				Proposed method				Alternative procedure			
	Inorganic Hg Added as $HgCl_2$											
Average residue	0.255				0.267							
Spike	0.00	0.20	0.40	0.60	0.00	0.20	0.40	0.60				
Residue + spike (theoretical)	0.255	0.455	0.655	0.855	0.267	0.467	0.667	0.867				
Actual recovery	0.266	0.464	0.680	0.890	0.266	0.459	0.645	0.880				
	Organic Hg Added as CH_3HgCl											
Amt sample digested, g	5.0				1.5				5.0			
Average residue	0.255				0.267				0.258			
Spike	0.00	0.200	0.40	0.60	0.00	0.20	0.40	0.60	0.00	0.20	0.40	0.60
Residue + spike (theoretical)	0.255	0.455	0.655	0.855	0.267	0.467	0.667	0.867	0.258	0.458	0.658	0.858
Actual recovery	0.244	0.464	0.652	0.875	0.267	0.460	0.660	0.865	0.264	0.480	0.660	0.862
Residue + spike (theor. total amt, μg)	1.275	2.275	3.275	4.275	0.40	0.70	1.00	1.30	1.290	2.290	3.290	4.290
Residue + spike (actual total amt recovered, μg)	1.220	2.320	3.260	4.375	0.40	0.69	0.99	1.298	1.320	2.400	3.300	4.310

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Table 2. Mercury contents (μg/g) of canned and frozen tuna and standard powders [a]

Munns and Holland (3)	Proposed method
Frozen Tuna	
0.040	0.033
0.090	0.093
0.136	0.146
0.140	0.150
0.146	0.150
0.223	0.240
0.232	0.235
0.232	0.233
0.236	0.238
0.239	0.224
0.240	0.236
0.248	0.260
0.248	0.233
0.362	0.370
0.442	0.456
Canned Tuna	
0.066	0.055
0.112	0.105
0.131	0.139
0.144	0.145
0.156	0.153
0.164	0.152
0.176	0.167
0.236	0.244
0.256	0.269
0.252	0.250
0.280	0.285
0.292	0.297
0.300	0.270
0.376	0.373
0.404	0.419
Fish Flesh Sample MA-A-2	
0.540	0.550
(0.54 ± 0.06 overall average (8))	
(0.49 ± 0.02 average after Dixon test (8))	
Dried Powder	
0.900	0.865
(0.857 ± 0.061 (7))	

[a] Averages of triplicate analyses.

temperature 10 min with occasional swirling. Place tube in rack and immerse to ca 12 mL mark in constant temperature water bath which is already adjusted to 95–100°C, and heat 20 min. Cool tube somewhat by immersing rack in cold water. Add 1 drop H_2O_2 (30%), mix tube by swirling, and dilute to 25 mL mark at room temperature. Pour solution into 150 mL flask used for mercury vapor generation and use 75 mL diluting solution (6) for complete transfer. Obtain standard graph and determine mercury content of solution as directed above.

Table 3. Reproducibility studies of 10 digestions of canned tuna sample (μg Hg/g)

Statistic	Munns and Holland (3)	Proposed method
Range	0.244–0.272	0.257–0.273
Mean	0.255	0.267
SD	0.012	0.015

Modified Procedure

Weigh 5.0 g sample in same flask used in Munns and Holland (3) method, add 20–25 mg V_2O_5 and 20 mL H_2SO_4–HNO_3 (1 + 1), and leave at room temperature 10 min with occasional swirling. Heat flask in water bath at 95–100°C 20 min, cool under tap, and add 15 mL water, 2 drops of H_2O_2 (30%), and 15 mL water. Let cool, and transfer solution to 100 mL volumetric flask. Dilute to volume and determine Hg content on 25 mL aliquot as directed above.

Results and Discussion

Our purpose in this study was to investigate the merits of open digestion in the determination of mercury in fish by the collaboratively studied method of Munns & Holland (3), which is also an alternative AOAC method (6). The Munns and Holland procedure specifies a 16 min reflux digestion performed with a Bunsen burner but our preliminary studies revealed that this digestion could not be performed in the *open* because of the onset of charring after approximately 7 min. The method could be modified by substituting a water bath at 95–100°C. In the proposed open digestion procedure, a smaller amount of sample is digested in a calibrated 50 mL Nessleriser tube, and the solution is diluted to volume (25 mL) in the tube itself, thus avoiding the transfer of solution to a volumetric flask. In addition, all of the prepared solution is used for mercury vapor generation; solution is completely transferred by washing with diluting solution. The proposed method allows as many as 40 digestions to be carried out simultaneously compared with about 6 by the reflux digestion technique.

Recovery studies were performed by spiking a tuna sample which, upon repeated analysis by the Munns and Holland (3) procedure, gave an average Hg content of 0.255 μg/g. The sample was then spiked with suitable aliquots of 1 μg Hg/mL aqueous solutions of $HgCl_2$ and MeHgCl. Results are given in Table 1.

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Table 4. Effect of digestion time on recoveries of Hg (added as CH_3HgCl) in proposed method

Recovery	Sample	Sample + 0.20 ppm spike	Sample + 0.4 ppm spike	Sample + 0.6 ppm spike
Theoretical	0.267	0.467	0.667	0.867
After 15 min	0.258	0.479	0.645	0.854
After 25 min	0.260	0.472	0.660	0.875
After 50 min	0.265	0.460	0.662	0.867

For comparison studies, digestion by both methods were performed in triplicate on 15 samples each of frozen and canned tuna and 2 standard powders (7, 8). Mercury content of the resulting solutions was determined by cold vapor technique (3, 6), and the results are reported in Table 2. There is good agreement between the reflux digestion and the proposed open digestion.

The proposed method was tested for reproducibility by making 10 digestions of a canned tuna sample. Results compared with the original procedure are given in Table 3. In the proposed method, a smaller amount of sample and added mercury is used; therefore, recoveries of organic mercury were also performed by the alternative procedure in which the total amount of sample and added mercury are the same as those used by Munns and Holland (3) in their recovery studies.

The effect of digestion time on recovery of organic mercury was also examined to determine if losses occur with prolonged heating. However, no losses occurred with heating times of up to $2\frac{1}{2}$ times the normal 20 min (Table 4).

In the acid digestion/cold vapor technique, cleaning and rinsing of glassware is an essential but laborious part of the analysis. The proposed open digestion technique not only reduces the amount of glassware, it offers a fast and simple approach to sample digestion, dilution, and transfer. It also enables us to analyze at least 5 times as many samples as with the current AOAC method.

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(8) International Laboratory of Marine Radioactivity (IAEA) (1980) Intercalibration of Analytical Methods of Marine Environmental Samples, Progress Report No. 20, Musee Oceanographique, Principality of Monaco

FOR YOUR INFORMATION

AOAC Welcomes Private Sustaining Members

AOAC Bylaws were changed at the 94th Annual Meeting in October 1980 to establish Private Sustaining Memberships for Industry.

By June of this year, seven companies had become AOAC Private Sustaining Members: the Fertilizer Institute, Washington, DC; International Minerals and Chemicals (IMC) Corporation, Northbrook, IL; ICI Americas, Inc., Goldsboro, NC; Eastman Chemical Products, Inc., Kingsport, TN; Pennwalt Corp., Agrochemicals Div., Philadelphia, PA; The Upjohn Co., Kalamazoo, MI; and Alcon Laboratories, Inc., Forth Worth, TX.

ISO Standards Available From ANSI

The following is a list of standards from the International Organization for Standardization (ISO), Technical Committee 34-Agricultural Food Products. The Standards are available at prices indicated from American National Standards Institute, Inc., 1430 Broadway, New York, NY 10018.

ISO 882-1980	Cardamoms—Specification	$ 8.40
ISO 973-1980	Spices and condiments—Pimento (allspice), whole, or ground—Specification	8.40
ISO 1003-1980	Spices and condiments—Ginger, whole, in pieces, or ground—Specifications	10.50
ISO 1546-1981	Procedure for milk recording for cows	12.60
ISO 1841-1981	Meat and meat products—Determination of chloride content (Reference method).	8.40
ISO 2255-1980	Coriander, whole or ground (powdered)—Specification	8.40
ISO 3632-1980	Saffron—Specification	14.70
ISO 5548-1980	Caseins and caseinates—Determination of lactose content—Photometric method	8.40
ISO 5553-1980	Meat and meat products—Detection of polyphosphates	8.40
ISO 5559-1981	Dehydrated onion—Specification	10.50
ISO 5560-1981	Dehydrated garlic—Specification	10.50
ISO 5738-1980	Milk and milk products—Determination of copper content—Photometric reference method	10.50
ISO 6091-1980	Dried milk—Determination of titratable acidity (Reference method)	6.30
ISO 6495-1980	Animal feeding stuffs—Determination of water-soluble chlorides content	8.40
ISO 6540-1980	Maize—Determination of moisture content (on milled grains and on whole grains)	16.80
ISO 6645-1981	Wheat flour—Determination of dry gluten	6.30
ISO 6660-1980	Mangoes—Guide to storage	8.40

Meetings

August 11-14, 1981: Symposium on Cereals: A Renewable Resource, Carlsberg Research Center, Copenhagen, Denmark. Cosponsored by the American Association of Cereal Chemists and the Carlsberg Research Center. The 3½-day session will feature technical presentations in which the basis for production and utilization of cereals will be reviewed. Fees: $195 for participants and $90 for spouses. For more information, contact Dorothy Ginsburg, AACC, 3340 Pilot Knob Road, St Paul, MN 55121; telephone 612/454-7250.

August 16-22, 1981: 28th Congress of the International Union of Pure and Applied Chemistry, the University of British Columbia, Vancouver, BC, Canada. Recent advances in

energy, the environment, resource development, oceanography, extraction of metals, soils chemistry, pulp and paper, corrosion, medicinal and drug chemistry, and biotechnology will be reviewed. For more information, contact Congress Secretariat, c/o The Chemical Institute of Canada, 151 Slater St, Suite 906, Ottawa, Ontario, Canada K1P 5H3; telephone 613/233-5623.

October 19–22, 1981; 95th Annual AOAC Meeting, Marriott Hotel, Twin Bridges, Washington, DC. Current developments in analytical methodology pertaining to agricultural, environmental, and public health areas will be presented and discussed. Five symposia are planned: AOAC Methods Development—the Challenge of the Next Decade, The Computer in the Laboratory, Infant Formula Regulation and Infant Food Problems, Trichothecenes, and Analytical Methodology for Lead in Foods. About 200 papers will be given on new techniques, methods, and instrumentation for analysis of foods, drugs, pesticides, cosmetics, feeds, fertilizers, mycotoxins, beverages, colors, forensic science materials, hazardous substances, vitamins, water and air pollutants, microbiological and extraneous materials contamination of foods, and related subjects. For further details, contact Kathleen Fominaya, AOAC, 1111 N. 19th St, Arlington, VA 22209; telephone 703/522-3032.

November 1–5, 1981: 109th American Public Health Association Annual Meeting, Los Angeles, CA. The general theme of the meeting will be "Energy, Health, and the Environment." For more information, contact Jon M. Counts, Bureau of Laboratory Services, Arizona Department of Health Services, 1520 W. Adams St, Phoenix, AZ 85007; telephone 602/255-1188.

April 13–15, 1982: 7th Annual AOAC Spring Training Workshop and Exposition, Fairmont Hotel, New Orleans, LA. Planned for this workshop are sessions on: drugs and antibiotics in feeds, food toxicology, forensic chemistry, pesticide residues, seafood quality, mycotoxins, environmental monitoring, fertilizers, sugars, laboratory automation, pesticide formulations, quality assurance, veterinary toxicology, hazardous waste monitoring, and analysis of toxicological substances. For additional information, contact co-chairmen: Nicole F. Hardin, U.S. Food and Drug Administration, 4298 Elysian Fields Ave, New Orleans, LA 70122; telephone 502/589-2471, or Hershel Morris, Louisiana Department of Agriculture, PO Box 16390-A, University Station, Baton Rouge, LA 70893; telephone 504/388-2755.

Three Methods Adopted Interim First Action

The following methods have been approved by the respective General Referees and Committees for adoption as interim official first action. The Association will vote on adoption as official first action at the 95th Annual Meeting, Oct. 19–22, 1981.

Infrared Spectrophotometric Method for Methazole Formulations, John E. Forrette, Velsicol Chemical Corp., Chicago, IL

Protein Nitrogen Unit Precipitation Procedure for Allergenic Extracts, Joan C. May and Jenny T. C. Sih, Food and Drug Administration, Bethesda, MD

Internal Insect Infestation of Bulk Wheat, Richard L. Trauba, Food and Drug Administration, Minneapolis, MN

Preprints of these methods are available from the AOAC office, 1111 N 19th St, Suite 210, Arlington, VA 22209.

BOOK REVIEWS

Colorimetric Chemical Analytical Methods. 9th Ed. By L. C. Thomas and G. J. Chamberlin. The Tintometer Ltd, Salisbury, UK; John Wiley and Sons Ltd, New York, NY, 1980. x + 625 pp. Price $85.00.

Chemical tests developed for use with several types of Lovibond color comparing equipment are the subject of this book.

It should be useful to industrial and regulatory agency chemists when screening tests for a wide range of compounds are required but a high degree of accuracy is not. Research chemists should find many of the isolation techniques useful in developing "cleanup procedures."

Each method is briefly and clearly written and is followed by an adequate list of references. The largest section covers the determination of metals, trace elements, and commonly found inorganic compounds, such as phosphate, nitrates, and hydrogen sulfide, in biological materials, foods, fuels, lubricants, soils, ores, and plating baths. A large section is also devoted to toxic substances, such as phosgene, acetone, nitrogen dioxide, and mercury, often found in air in industrial and urban atmospheres. A smaller section covers several organics commonly found in water, sewage, and biological materials. A section on biochemistry, pathology, and pharmacology includes several techniques used in hematology, clinical chemistry, and urinalyses for determining levels of urea, transaminase, phosphorus, and cholesterol. A final section covers a wide variety of color grading and quality tests primarily for foods and water.

Overall, the book is well written and provides a large number of simple, rapid methods for a wide range of compounds in many common matrices.

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Midland Macromolecular Monographs, Volume 7, Silylated Surfaces. Edited by Donald E. Leyden and Ward T. Collins. Gordon and Breach Science Publishers, New York, NY, 1980. 379 pp. Price $63.50.

The use of silylated surfaces in analytical applications is most often associated with chromatographic techniques. This book, relating the proceedings of the May 1978 Midland Macromolecular Institute Symposium on Silylated Surfaces, offers valuable insights into other areas where such surfaces prove useful, such as: electrochemistry, enzyme immobilization, catalysis, and trace materials enrichment techniques. To provide an interdisciplinary exchange of information on the utility of silylation reactions, the organizers assembled a variety of contributors. Valuable inclusions are a collection of papers describing several methods for the analysis of surfaces and discussions of secondary ion mass spectrometry (SIMS), Fourier transform infrared (FTIR), UV resonance Raman spectroscopy, and x-ray photoelectron spectroscopy (ESCA).

The first two chapters discuss the chemistry of glass surfaces and of silane coupling agents, providing an ideal background for the topics discussed later in the book. The immobilizing of dye molecules of aminosilanes to electrode surfaces for unique electrochemical applications is shown to be a relatively new area of research with many problems yet to be solved. Reverse phase liquid chromatography with alkylsilanes bonded to silica supports is extensively reviewed. The possible applications of more unusual forms of liquid chromatography, such as with charge-transfer bonded phases and ligand exchangers, are also considered. The chapters on the characteristics and performance of immobilized enzyme supports present excellent discussions of the basic principles involved in this increasingly important technology.

This monograph is a good overview of silylated surfaces and their uses. No subject is covered in great depth, but good introductions to several related areas of technology are included.

JOSEPH J. DESTEFANO
E. I. du Pont de Nemours & Co.
Analytical Instruments Division
Wilmington, DE 19898

NEW PUBLICATIONS

Analytical Methods for Pesticides and Plant Growth Regulators. Volume XI, Updated General Techniques and Additional Pesticides. Edited by Gunter Zweig and Joseph Sherma. Published by Academic Press, 111 Fifth Ave., New York, NY 10003, 1980. 416 pp. Price $46.00, subscription price $39.00. ISBN-0-12-784311-6.

The present volume, the eleventh in this treatise, now in its 17th year of publication, is devoted to updating analytical methods and adding pesticides not previously included. One chapter presents an update on the automated pesticide analytical laboratory and presents techniques that are readily available in addition to some that are still on the drawing board. This chapter points to the time when sample extraction, cleanup, injection into the chromatographic column, and final calculations may be fully automated. Other chapters discuss analysis of *N*-nitroso compounds, analytical methods for formulations and residues of recently developed pesticides, analysis of ethylenebisdithiocarbamate (EBDC) fungicides and degradation products, and analytical methods for the toxicologically important degradation product ethylene thiourea (ETU).

Test Protocols for the Environmental Fate and Movement of Toxicants. Proceedings of a 1980 AOAC Symposium, Washington, DC, October 21–22, 1980. Published by the Association of Official Analytical Chemists, 1111 N 19th St, Arlington, VA 22209, 1981. 336 pp. Price $30.00 U.S., $33.00 all other countries. ISBN-0-935584-20-X.

Since the introduction of synthetic pesticides scientists have endeavored to understand how these chemicals degrade and how parent compounds and degradation products are transported throughout the environment. Out of this research has evolved a series of environmental chemical and biological tests. The 17 papers included in this book provide the latest protocols for specific environmental tests and methods for interpreting or predicting the results by the use of mathematical models. Subjects of papers include tests for physical and chemical properties, for mobility in soil and water, for metabolism—accumulation/degradation, and for microbial metabolism; field dissipation studies; and mathematical modeling of the fate and movement of toxicants in ecosystems, of toxicant transport through soil, and of environmental assessment of toxicants.

Physical Methods in Modern Chemical Analysis. Volume 2. Edited by Theodore Kuwana. Published by Academic Press, 111 Fifth Ave., New York, NY 10003, 1980. 432 pp. Price $45.00. ISBN-0-12-430802-3.

The chapter subjects of this, the second volume in a series on physical/instrumental methods of chemical analysis are: high performance liquid chromatography, x-ray photoelectron spectroscopy, x-ray diffraction methods applied to powders and metals, analytical aspects of ion cyclotron resonance, and refractive index measurement. Each chapter covers the theory and principles on which the particular method is founded, gives a description of the instrumentation or equipment used, and illustrates the scope and power of the method by presenting several selected examples.

Physical Principles of Pesticide Behavior—The Dynamics of Applied Pesticides in the Local Environment in Relation to Biological Response. Volume 2. By G. S. Hartley and I. J. Graham-Bryce. Published by Academic Press, 111 Fifth Ave., New York NY 10003, 1980. 524 pp. Price $74.50. ISBN-012-328402-3.

The objective of the authors of this book was to provide a text on the properties and processes of pesticides—volatility, solubility, partition, adsorption, diffusion, flow, impaction and spreading—which determine behavior in the immediate environment of their application. The book is mainly addressed to research workers with a physico-chemical training, to those concerned with the design and testing of new formulations and methods of application, and should be of interest to field workers, biochemists, and environmentalists. Chapter subjects include: the relation of permeation to toxicity, penetration of pesticides into higher plants and into insects and fungi, effects of growth and movement of organisms on interception of pesticides, and application and formulation.

Instrument Methods of Analysis. Sixth Edition. By Hobart H. Willard, Lynne L. Merritt, Jr., John A. Dean, and Frank A. Settle, Jr. Published by Van Nostrand Reinhold, 135 W 50th St, New York, NY 10020, 1981. Approx. 950 pp. Tentative price $27.95. ISBN 0-442-24502-5.

Emphasizing both instrumentation and the methodology that employs it, this new edition remains a single-volume book with extensive coverage of instrumental analysis. All significant chromatographic methods are included. A series of chapters on microprocessor-controlled instrumentation has been added.

Nickel Toxicology. Proceedings of the Second International IUPAC Conference on Nickel Toxicology, Swansea, Wales, September 3–5, 1980. Edited by Stanley S. Brown and F. William Sunderman, Jr. Academic Press, Inc., 111 Fifth Ave, New York, NY 10003, 1980. 214 pp. Price $36.00. ISBN-0-12-137680-X.

In the past few years, there has been a remarkable increase in our understanding of the toxicology of nickel and its compounds. This book provides the current research on important aspects of nickel toxicology and gives a perspective of the chemistry and biology of nickel. Chapter subjects include epidemiological aspects; experimental approaches; uptake, distribution and excretion; pharmacologic effects; and analytical aspects. The book should be of special interest to toxicologists, cancer epidemiologists, occupational physicians, and hygienists; to individual and governmental agencies concerned with nickel manufacture and processing; and to biochemists, nutritionists, and pharmacologists who work with trace metals.

Wilson and Wilson's Comprehensive Analytical Chemistry. Edited by G. Svehla. Volume XII—Thermal Analysis, Part A: Simultaneous Thermoanalytical Examinations by Means of the Derivatograph. By J. Paulik and F. Paulik, 1981. Published by Elsevier Scientific Publishing Company, PO Box 211, 1000 AE Amsterdam, The Netherlands; Elsevier North-Holland, Inc., 52 Vanderbilt Ave, New York, NY 10017. xviii + 278 pp. Price US $73.25/Dfl. 150.00. ISBN-0-444-41949-7.

Part A gives a history of the simultaneous thermoanalytical technique and methods that have been developed for its use. Thermogas titrimetry, a dilatometric measuring technique, and the quasi-isothermal–quasi-isobaric methods are discussed as are more general aspects, such as the influence of experimental conditions, resolution and selectivity of the examinations, and information on the multiplying effect of simultaneous examinations. A list of about 1400 publications reporting results obtained by means of the derivatograph and a subject index of 1100 compounds are included.

Nondestructive Activation Analysis With Nuclear Reactors and Radioactive Neutron Sources. Studies in Analytical Chemistry 3. Edited by S. Amiel. Published by Elsevier Scientific Publishing Company, PO Box 211, 1000 AE Amsterdam, The Netherlands; Elsevier North-Holland, Inc., 52 Vanderbilt Ave, New York, NY 10017, 1981. xvi + 363 pp. Price U.S. $83.00/Dbl. 170.00. ISBN-0-444-41942-X.

The many recent developments in detection instrumentation (Ge(Li) and Si(Li) detectors) have stimulated interest in the use of nondestructive activation analysis. This book is a guide describing when and how this technique may be used to produce optimally sensitive and accurate results. The sequence of the text follows that of the principal steps in nondestructive activation analysis. Brief descriptions of the basic principles are included for the inexperienced reader.

Although analytical chemists and nuclear chemists are the primary audience for this book, for other disciplines information has been included on geo- and cosmochemical archaeological, forensic science, environmental, biomedical, and industrial applications of the technique.

Systematic Identification of Organic Compounds. A Laboratory Manual, 6th Edition. By Ralph L. Shriner, Reynold C. Fuson, David Y. Curtin, and Terence C. Morrill. Published by John Wiley and Sons, Inc., Dept 5824, Somerset, NJ 08873, 1980. 604 pp. Price $22.95.

A new edition of this laboratory manual offers important, up-to-date, reliable

techniques to help in the identification of organic compounds. The book lays out explicit analytical procedures, operating instructions for laboratory instruments, integrating spectroscopy, and spectral problems. All chapters have been rewritten to reflect recent advances. New material includes special characterization techniques and references, new tables that describe the composition and properties of common reagents, solvent classification techniques to aid functional group identification, "wet" tests for nitrogen and various functional groups, and modern analytical techniques.

Personal Computers in Chemistry. Edited by Peter Lykos. Published by John Wiley and Sons, Inc., Dept 5824, Somerset, NJ 08873, 1980. 280 pp. Price $27.50.

A series of 20 technical papers on applications of microprocessor-based systems for chemists comprise this book. The papers cover computer use in research, management, education, and writing; they discuss costs, reliability, maintenance, graphic display, operating systems, languages, and designing a system for your own work. The applications cover a wide range from the use of a small personal computer for collecting data to an interactive laboratory data system. Computers capable of speech generation and recognition and a device that enables you to use many mathematical tools more easily are also discussed.

CORRECTION

J. Assoc. Off. Anal. Chem. (1981) **63**, 91–103, Free Sugars and Sorbitol in Fruits—A Compilation from the Literature, by R. E. Wrolstad and R. S. Shallenberger; Table 2, p. 93:

The second sample listed should read "USA (applesauce)"

Analytical Proceedings

Following the development and expansion of the former *Proceedings of the Analytical Division of The Chemical Society* to include a wide range of topics of general interest to analytical chemists, a change of title to

ANALYTICAL PROCEEDINGS

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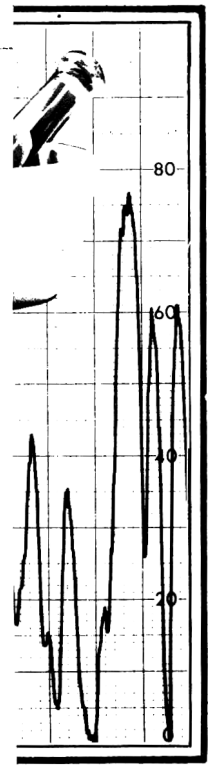

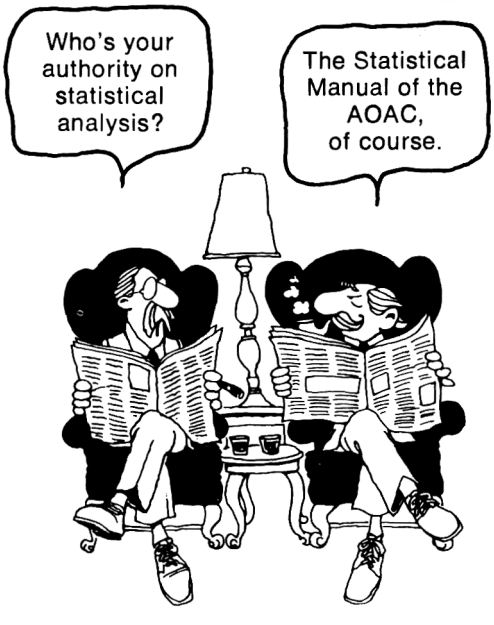

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