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ALCOHOLIC BEVERAGES

Thin Layer Densitometric Determination of Gallic Acid and Gallotannins in Wine and Cider

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Free gallic acid and total gallotannins are determined in wine and cider by thin layer densitometry. The beverage is extracted with ethyl acetate and the extract is subjected to thin layer chromatography on silica gel. After elution with chloroform–ethyl formate–formic acid (50+40+10), the dry plate is sprayed uniformly with a freshly prepared solution of 0.3% aqueous $FeCl_3$ and 0.3% aqueous K_3FeCN_6. If the gallic acid spot appears (R_f 0.28), densitometry readings are taken at 600 nm, and free gallic acid (GA_F) is calculated from the formula GA_F = GA (μg, from calibration curve) × 1.25. The calibration curve was obtained by plotting thin layer densitometric readings at 600 nm (prussian blue) vs. μg gallic acid. In a second method, the beverage is hydrolyzed, and extraction and densitometry are performed as before to give "total gallic acid after hydrolysis:" GA_H (mg/L) = GA (μg, from calibration curve) × 25. Total gallotannins ($GALL_T$) are calculated from the formula: $GALL_T$ (mg/L) = $GA_H - GA_F$. Results for 10 wines and 5 ciders are presented and briefly discussed.

The occurrence and role of gallic acid and hydrolyzable tannins (gallotannins and ellagitannins) in wine and grape has been discussed in detail (1–3). Gallic acid in red Cabernet Sauvignon, for example, was reported to be 10 mg/L; in grape skin or white wine, 0–0.5 mg/L; and 12 mg/L before or after saponification of a red wine. Free gallic acid was found in wines just after fermentation, particularly if fermented on the skins. It was reported absent in aged wines, Riesling grapes, or grapeseed extracts containing depside gallates (1–3).

Esters of gallic acid are present in grapes and wine. They undergo autooxidation to ellagic acid more readily than does gallic acid itself. This interconversion is relevant to the presence of hydrolyzable tannins (gallotannins and ellagitannins) in wine. Hydrolyzable tannins, however, do not occur in wine or grapes in large amounts (2), and the "true tannins" in these substrates are condensed tannins (flavan-derived). This view was supported by application of Stiasny's formaldehyde reagent, which precipitates condensed tannins but not gallotannins. A further proof was Dadic's spectrophotometric determination of condensed tannins and their precursors (tanninogens, polyhydroxyflavans), which accounted for nearly the total phenolic content of wine (2, 4).

The presence of gallotannins in wine or cider may be due to the use of tannic acid as a chillproofing agent and antioxidant. Wood barrels, particularly those made of white oak, are an additional source of hydrolyzable tannins as well as other phenolics in wine. These phenolics include lignins and their fragments (gallic and ellagic acids), as well as the aldehydes and acids related to vanillin. Ellagitannins, however, are the major phenolic components of oak wood. Wine, with its alcoholic content, extracts more tannin than lignin from oak barrels, and about 60% as much total solids as does brandy, for example. Longer storage will contribute more wood phenolics to wine.

Tannins, and to a lesser degree gallic acid, were reported to intensify the red color of wine. Gallic acid as well as caffeic and quinic acids stimulated somewhat the wine-yeast fermentation (at 100 mg/L). Hydrolyzable tannins, on the other hand, retarded or suppressed completely the fermentation (at 2,000–4,000 mg/L). Tannins contribute significantly to wine flavor, particularly to its astringency and bitterness.

In this communication, a thin layer densitometric method for quantitative determination of gallic acid and gallotannins in wine and

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cider is described. Thin layer chromatography was first used by Dadic and Belleau (5) to determine these phenolic substances in beer.

METHODS

Apparatus and Reagents

(a) *Spectrodensitometer.*—Schoeffel Model SD 3000. Use deionized double-distilled water.

(b) *Silica gel 60 plates.*—0.25 mm (EM Laboratories, Inc., Elmsford, NY 10523).

(c) *Silica gel G plates.*—0.25 mm (Analtech Inc., Newark, DE 19711).

(d) *Volupettes.*—Dade Division American Hospital Supply Corp., Miami, FL 33152.

(e) *Solvents.*—Glass-distilled, spectroscopic, or chromatographic grade ethyl acetate, methanol, ethanol, chloroform, ethyl formate, and formic acid.

(f) *$FeCl_3$ and K_3FeCN_6.*—Reagent grade. Prepare 0.3% aqueous solutions.

Determination of Free Gallic Acid (GA_F)

To 40 mL wine (or cider), add 40 mL 6*N* HCl and 120 mL deionized water. Shake mixture with 150 mL ethyl acetate 5 min in separatory funnel. Shake aqueous layer with additional 75 mL ethyl acetate, and dry combined organic layers over 30 g anhydrous sodium sulfate. Remove solvent under reduced pressure at room temperature, and extract residue with 10–20 mL ether (or methanol). After decantation and removal of ether, dissolve residue in 100 μL ethanol. Spot 2 μL ethanolic solution on prescored silica gel 60 plate (0.25 mm layer thickness), using syringe or Volupette. (If several samples are analyzed, spot in alternate channels of prescored plate, in case densitometry has to be performed.) Apply spots 3 cm from bottom of plate and let migrate 15 cm, using chloroform–ethyl formate–formic acid (50+40+10) as eluant. Dry plate in vacuum desiccator and spray uniformly with freshly prepared solution of 0.3% aqueous $FeCl_3$ and 0.3% aqueous K_3FeCN_6, mixed just before use.

If blue gallic acid spot appears (R_f 0.28), take densitometric readings at 500 nm, and read amount of gallic acid in sample from calibration curve. Take average value of several readings. Obtain calibration curve by plotting thin layer densitometric readings at 600 nm (prussian blue) against amounts of gallic acid in μg (0.1–1.2 μg).

$$GA_F\ (mg/L) = GA\ (\mu g) \times 1.25$$

Determination of Total Gallotannins ($GALL_T$)

To 40 mL wine (or cider), add 40 mL 6*N* HCl and 120 mL water. Reflux mixture 7 hr at refluxing rate of 50–70 drops/min. (Hydrolysis should be carefully controlled because too harsh conditions may lead to disappearance of blue spot at R_f 0.28.) After cooling to room temperature, proceed with ethyl acetate extraction as described above, and dissolve residue (after drying and removing solvent) in 2 mL ethanol. Use 2 μL ethanolic solution for thin layer chromatography and thin layer densitometry as described above.

Total gallotannins are then calculated from "gallic acid after hydrolysis" (GA_H) and from free gallic acid (GA_F) as follows:

$$GA_H\ (mg/L) = GA\ (\mu g) \times 25$$

$$GALL_T\ (mg/L) = GA_H - GA_F$$

Results and Discussion

The overwhelming portion of phenolic compounds in wine consists of condensed tannins and their precursors (tanninogens) (2, 4), as determined by spectrophotometric determination of these substances (tanninogens). Experimental results submitted in this communication represent additional proof: The gallotannin concentrations in wine equalled only a small fraction of its total phenolic content (2, 4).

The amounts of endogenous gallotannins in wine and cider usually exceed 1 mg/L. Thus, the described method does not distinguish endogenous gallotannins ($GALL_E$) and those (possibly) added as "tannic acid" ($GALL_A$) during processing of the beverage. This differentiation was possible for beer, which does not contain endogenous gallotannins in amounts exceeding 1 mg/L (5).

The method quantitatively determines free gallic acid (GA_F) and total gallotannins ($GALL_T$) in wine and cider, using thin layer chromatography. Visualization of thin layer chromatographic (TLC) plates is performed with $FeCl_3/K_3FeCN_6$ reagent and is based on the gallic acid reduction of Fe^{+++} to Fe^{++}, resulting in prussian blue formation. A representation of a typical thin layer chromatogram for a red wine, a rosé wine, a white wine, a cider, as well as reference gallic and tannic acids is given in Fig. 1. The gallic acid spot in the examined beverages is free from interference and can be easily measured by thin layer densitometry.

Free gallic acid is a sum of endogenous gallic acid (GA_E) and gallic acid added (GA_A), i.e., when tannic acid is used in processing the beverage. Tannic acid, used in processing beer as well as wine and cider, usually contains less than 8% of the free gallic acid monomer (5). Naturally, if no tannic acid is used in processing the beverage, $GA_A = 0$, and $GA_F = GA_E$.

Total gallic acid after hydrolysis (GA_H) is

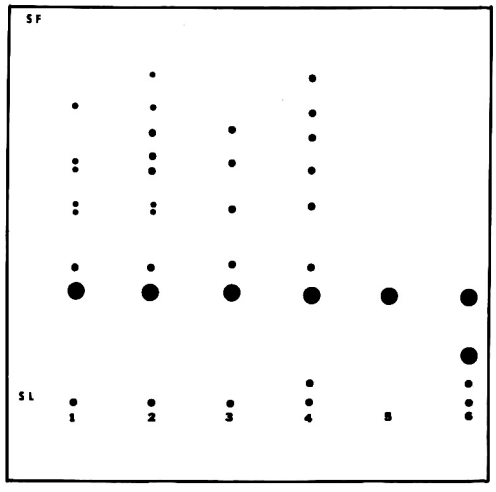

FIG. 1—TLC pattern for a red wine (1), a white wine (2), a rosé wine (3), a cider (4), and reference gallic (5) and tannic (6) acids. SL = starting line; SF = solvent front.

determined by thin layer densitometry, whereby

$$GA_H = GA_F + GA_{GALL}$$

GA_{GALL} represents gallic acid moieties of gallotannin molecules. Various molecular ratios for gallic acid moieties in natural gallotannins were proposed, ranging from $GA_{GALL}/GALL = 0.81–1.0$ (6). For simplicity, this factor has been chosen to equal 1. Thus, total gallotannins in beverage can be calculated by using a simple formula:

$$GALL_T = GA_H - GA_F$$

$$(GALL_T = GA_{GALL}, \text{ for } GA_{GALL}/GALL = 1)$$

Calculations for GA_F and $GALL_T$, in mg/L (or ppm), are performed by using the equations:

$$GA_F \text{ (mg/L)} = GA \text{ } (\mu g) \times 1.25$$

$$GALL_T \text{ (mg/L)} = GA_H - GA_F$$

where GA_H (mg/L) = GA (μg) × 25

Note: Factors 1.25 and 25 derive from the analytical procedure, which specifies 40 mL sample and 2 μL portion of a 100 μL total extract, or 2 μL portion of a 2 mL total extract.

The calibration curve for beer (5) was obtained by adding tannic acid to a beer not treated with tannic acid and, consequently, devoid of gallotannins (within the sensitivity of the method, 1 mg/L). This eliminated the need for using the $GA_{GALL}/GALL$ factor. The same procedure cannot be followed with wine and cider, which normally contain endogenous gallotannins in excess of 1 mg/L.

Recovery tests were performed by adding tannic acid to the beverages (in the concentration range of interest); recovery was greater than 95%.

A positive prussian blue visual test for digallic acid (DIGA, R_f 0.12) could be used as an indicator that gallotannins (tannic acid) were used in beverage processing, if the assumption is made that no endogenous digallic acid ($DIGA_E$) is present in wine and cider. This assumption still remains to be proven experimentally.

Results for 10 wines and 5 ciders are presented in Table 1. All the analyses were done in replicate and the average values are given. The experimental error was ± 5%.

Free gallic acid (GA_F) in wines ranged from 0.3 to 2.0 mg/L (average of 10 samples: 1.1 mg/L). Red wines were the richest (average of 4 samples: 1.7 mg/L), and white wines the poorest (average of 4 samples: 0.7 mg/L) in GA_F content. Rosé wines (average of 2 samples: 0.8 mg/L) were in between, but closer to the GA_F content of white wines.

GA_F of ciders ranged from 0 to 0.5 mg/L (average of 5 samples: 0.3 mg/L). Thus, it appears that ciders are generally poorer in free gallic acid than are wines.

Total gallotannins, $GALL_T$, in wines ranged from 1.2 to 33.0 mg/L (average of 10 samples: 9.8 mg/L). Red wines were again the richest (average of 4 samples: 18.9 mg/L), and white wines the poorest (average of 4 samples: 3.3 mg/L) in $GALL_T$ content. Rosé wines (average of 2 samples: 4.8 mg/L) were in between, but closer to the $GALL_T$ content of white wines.

Thus, this limited survey (10 wines) shows that the same trend exists for free gallic acid (GA_F) and total gallotannins ($GALL_T$) contents as the one established for condensed tannins and their precursors (tanninogens) (4), i.e, red wines > rosé wines > white wines. This trend, however, should be confirmed on a greater number of wines.

Total gallotannins ($GALL_T$) in ciders could not be measured because hydrolysis produced a blue streak rather than discrete spots (prussian blue), making densitometry of gallic acid unfeasible. Whether this phenomenon is due to the particular nature of ciders analyzed in

Table 1. Gallic acid and gallotannins (mg/L or ppm) in wine and cider

Sample	Visual test (prussian blue) GA (R_f 0.28)	DIGA (R_f 0.12)	GA_F[a]	GA_H[b]	$GALL_T$[c]
Red wine A—Spain (Mosen Cleto)	+	+	2.0	22.5	20.5
Red wine B—Italy (Chianti)	+	+	2.0	35.0	33.0
Red wine C—Yugoslavia (Kastelet)	+	+	1.4	17.5	16.1
Red wine D—France (Mommesin)	+	+	1.4	7.5	6.1
White wine A—France (Cuvee des Patriotes)	+	+	0.5	4.2	3.7
White wine B—Germany (Wein Prinz)	+	+	0.5	1.7	1.2
White wine C—Greece (Sta. Helena)	+	+	1.7	6.6	4.9
White wine D—Portugal (Casal Garcia)	+	+	0.3	3.7	3.4
Rosé wine A—Portugal (Faisca)	+	+	1.0	5.0	4.0
Rosé wine B—Italy (Rosatello)	+	+	0.6	6.2	5.6
Cider A (10% alcohol)—Quebec (La Vielle Cuvee)	+	+	—	—	—
Cider B (10% alcohol)—Quebec (Moussablon)	+	+	0.2	—	—
Cider C (10% alcohol)—Quebec (Saint Antoine)	+	+	0.4	—	—
Cider D (10% alcohol)—Quebec (Mere Superieure)	+	+	0.5	—	—
Cider E (10% alcohol)—Quebec (Petit Normand)	+	+	0.3	—	—

[a] GA_F (free gallic acid before hydrolysis), mg/L = 1.25 × μg GA.
[b] GA_H (total gallic acid after hydrolysis), mg/L = 25 × μg GA.
[c] $GALL_T$ (total gallotannins after hydrolysis), mg/L = GA_H − GA_F. (For further explanations, see text.)

this work, or to a peculiar nature of cider gallotannins in general, remains to be established.

Acknowledgment

Thanks are due to Molson Breweries of Canada Ltd for permission to release these results.

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FERTILIZERS

Argon Plasma Emission Spectrometry of Boron in Fertilizers: Comparison with Spectrometric and Distillation Procedures

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A much faster and equally accurate method is described for determining boron in fertilizers by argon plasma emission spectrometry at 249.67 nm. Results by the method are compared with results from a methyl borate distillation method and a modified azo-methine H spectrometric procedure. No significant differences in results were found among the methods studied.

Methods for determining boron have many shortcomings. The AOAC official final action method (1) and the methyl borate distillation method (2) are cumbersome and lengthy. The atomic absorption methods lack the necessary sensitivity, and the flame emission techniques require organic extractions to remove interferences. Even though the specific color reagent proposed by Wolf (3, 4) has the necessary sensitivity, the color development is time-dependent, and reproducibility is poor. Modifications of Wolf's procedure give more reproducible results but still require lengthy color development periods (5; P. C. Whittier, Department of Agriculture, Atlanta, GA). Significant design improvements have been made in the plasma jet since argon plasma spectrometry (APES) was first proposed (6) for the determination of boron in fertilizer, which prompted this new investigation.

For re-evaluation of APES techniques, a recently developed three-electrode, direct current argon plasma coupled to a high resolution echelle monochromator was used to determine boron in fertilizers. The plasma jet is formed between 2 spectrographic carbon anodes and a tungsten cathode in an inverted Y configuration. This design resulted in improved stability and better detection limits than previous dc argon plasmas.

Experimental

Reagents and Apparatus

(a) *Boron standard solutions.*—1000 μg boron/mL. Dissolve 5.7195 g H_3BO_3 in water and dilute to 1 L. Prepare working concentrations between 0 and 10 μg boron/mL that contain 3 mL HCl/100 mL (linear working range).

(b) *Ion exchange resin.*—Amberlite IR-120. In a column, treat with 500 mL 10% HCl, wash free of acid with distilled water, and air-dry.

(c) *Argon plasma spectrometer.*—SMI Spectraspan III (Spectrametrics, Inc., 204 Andover St, Andover, MA 01810).

Sample Preparation

Weigh 1.000 g fertilizer into 150 mL beaker, add 10 g ion exchange resin and 30 mL water, and stir with magnetic stirrer 3 min. Filter into volumetric flask that will result in final concentration between 0 and 10 μg boron/mL when diluted to volume. Retain ion exchange resin for regeneration. Adjust acid concentration to 3 mL HCl/100 mL, and compare emission intensities of samples and standard by APES at 249.67 nm.

Results and Discussion

A study was made to determine the effects of some of the ions commonly found in fertilizers on the determination of boron by APES. This study was used to evaluate the claim that APES is virtually free of chemical interferences.

The concentrations of ions tested represented a range that would be present when 1 g fertilizer is digested and diluted to 100 mL. The effects of adding the major ions (K as KCl, PO_4 as H_3PO_4, Ca as $CaCl_2$) are shown in Table 1. The effects of the minor ions are

Table 1. Effect of major ions in fertilizer digests on the recovery of 2.0 μg boron/mL

	Boron recovered, μg/mL, at ion concn:			
Ion	250 μg/mL	500 μg/mL	1000 μg/mL	2000 μg/mL
Ca	2.00	2.01	2.02	2.04
K	2.07	2.08	2.13	2.19
PO_4[a]	1.95	1.95	1.96	1.97

[a] Calculated as P_2O_5.

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Table 2. Effect of minor ions in fertilizer digests on the recovery of 2.0 μg boron/mL

Ion	Boron recovered, μg/mL, at ion concn: 25 μg/mL	50 μg/mL	100 μg/mL	200 μg/mL
Al	1.97	1.98	1.98	1.98
Fe	2.06	2.03	2.06	2.06
Mg	2.02	1.98	2.03	2.02
Na	2.05	2.01	2.04	2.06

shown in Table 2. The data show that the only significant enhancement is caused by potassium.

Melton *et al.* (6) claimed that addition of lithium completely masked the enhancement caused by alkali metals. Our tests show that the enhancement is completely masked by lithium when only boron and potassium are present, but it is not effective on fertilizer digests. This suggests that enhancement is caused by interactions, and this effect must be eliminated. Tests showed that a batchwise extraction using 10 g cation exchange resin was sufficient to remove more than 90% of the cations from 1 g fertilizer solution. This decreases the concentration of cations so that no significant interference in the determination of boron by APES is observed.

Boron was determined on samples from a recent collaborative study (P. C. Whittier) and on Magruder check samples by APES, distillation, and azo-methine H (5) spectrometric procedures. The results are shown in Table 3. When the results by the APES method are compared with results by the spectrometric and distillation procedures, the paired t-test shows no significant differences at the 95% confidence level, and likewise the correlation coefficients show no significant differences: APES vs. spectrometric, $R^2 = 0.99998$; APES vs. distillation, $R^2 = 0.99998$. Aspiration into the plasma of an acid extract of these samples with and without the lithium addition gave results that showed an average positive bias of approximately 10%; the bias is greater at the lower boron levels. The time required for a boron determination by APES is significantly less than that for the alternative methods. Boron can be determined on a group of 6 samples with ease in 0.5 hr, while the spectrometric and the distillation procedures require approximately 2 and 8 hr, respectively.

Table 3. Determination of boron in selected fertilizers

Sample	Boron, % APES	Spectrom.	Distn
15- 5-10	0.042	0.040	0.040
10-10-10	0.022	0.023	0.022
4-12-24	0.125	0.119	0.123
8-16-24	0.025	0.023	0.021
20-20- 5	0.005	0.005	0.006
5- 4- 8	0.086	0.086	0.088
20-20- 5	0.052	0.056	0.056
4-12-24	0.129	0.132	0.128
4-12-24	0.273	0.276	0.277
4-12-24	0.176	0.174	0.173
5-10-15	0.046	0.043	0.042
Mag No. 7605	0.051	0.050	0.050
Mag No. 7304	0.205	0.201	0.202
Mag No. 7509	0.071	0.068	0.073
Mag No. 7611	3.01[a]	3.01	2.96
Mag No. 7711	0.032	0.031	0.032
Paired *t*-test [$t_{0.05}(15) = 2.131$]	distillation vs APES, $t = 1.127$		
	APES vs spectrometric, $t = 1.209$		

[a] Fritted micronutrient mixture solubilized with acid before ion exchange treatment.

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SUGARS AND SUGAR PRODUCTS

Hydroxymethylfurfural and Honey Adulteration

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The value of the determination of hydroxymethylfurfural (HMF) in the detection of invert sirup adulteration of honey is examined. Analysis of 481 samples of extracted honey and 41 comb honeys from producers, and samples of honey before and after processing from 8 packers provided basic data for establishing guidelines for HMF content of honey. A sample containing 20 mg/100 g or more should be considered as possibly adulterated and subjected to additional analysis for confirmation of the presence or absence of adulteration. Extremely high (about 50 mg/100 g) values are conclusive, however.

Detection of invert sirup added to honey has been a problem for nearly a century. Addition of moderate amounts of invert sirup does not cause glucose and fructose levels to fall outside of the normal range for honey. Qualitative color tests used in years past depended on the detection of hydroxymethylfurfural (HMF), which was produced during the acid-catalyzed inversion of sucrose. Indeed, the 12th edition of *Official Methods of Analysis* of the AOAC includes a resorcinol test for commercial invert sugar (**31.138**). The general unreliability of such tests is indicated by a note: "Resorcinol test, when neg., may not be regarded as conclusive evidence of absence of com. invert sugar sirup in honey."

Problems and evaluations of these tests are discussed elsewhere (1). The description in 1955 (2) of 2 quantitative methods for HMF in honey stimulated interest in their use for evaluation of honey quality. Based essentially on data from over 1700 samples of honey imported into Germany and Switzerland, the honey standards of the Codex Alimentarius (3) included a maximum value for HMF in table honey of 4 mg/100 g. This value was selected to assure that table honey available in the participating countries is not denatured by heat, thus destroying health-giving properties they believed to be present. Honey with higher HMF content is relegated to the manufacturing trades at lower prices.

For many years honey has been known to contain HMF arising from action of normal honey acidity (av. pH 3.9) on fructose at ambient temperatures and at an accelerated rate during heat processing or storage at elevated temperatures. This caused early difficulties with qualitative tests for invert sirup adulteration and must be recognized in differentiating between normally processed honey and that containing added invert sirup.

Several of the important honey adulterants do not contain significant amounts of HMF. High fructose corn sirup (HFCS) and many conventional (non-fructose) corn sirups (CCS) are lower than processed honey in HMF. Other tests are required to detect their addition to honey.

From the studies of Schade *et al.* (4), Hadorn and Kovacs (5), Gautier *et al.* (6), Hadorn and Zürcher (7), White *et al.* (8), and Gonnet (9) on the production of HMF in honey by heat and storage, it is apparent that, as with all other aspects of honey chemistry, this area is characterized by extreme variability. No fixed formula can be devised to predict exactly the effect of storage and heating on HMF content of honey. White *et al.* (8) found a linear relationship between storage temperature of honey and the logarithm of the time required to accumulate a given amount of HMF. This finding was based on extensive storage studies of 3 honey samples and was used to estimate that about 3 times the heat exposure required to produce an HMF concentration of 4 mg/100 g honey is needed to produce an HMF level of 20 mg/100 g honey (1). Any guideline established for a permissible HMF level in honey cannot be the sole basis for condemnation as adulterated. Additional compositional evidence is required. The measurement of HMF is a relatively easy procedure with the new bisulfite method (10) which is intended as a screening procedure for questioned samples to reduce the need for complete carbohydrate analysis for demonstrating the presence of added invert sirup (11).

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A study was therefore conducted to determine the HMF levels of United States honey as produced by beekeepers and to estimate the effect of United States commercial processing on HMF content. The objective was to provide a practical maximum level of HMF beyond which a sample would be suspected of being adulterated with invert sirup, without discriminating against genuine honey abused by heat or storage. A collection of 480 samples of United States honey, certified as genuine by their producers, was obtained for another purpose (12), and we determined the HMF content of these samples to obtain baseline values for such honey. We also analyzed honey in the comb to establish baseline values for the HMF content of unprocessed honey. By enlisting the cooperation of a number of honey packers, we also analyzed samples of honey before and after normal processing to obtain information on the increase of HMF caused by thermal processing.

Experimental

Producer Samples

Samples were from the 1974 and 1975 crop years, and information on floral type, heating, and storage history was provided for most of the 480 samples that were voluntarily submitted. Samples were refrigerated immediately when received and were not heated before HMF analysis.

Processing Samples

Packers, whose joint output represents at least 70% of United States commercial honey, were requested to provide samples of honey before and after their customary processing for both retail and bulk pack. This was done because circumstances prevented visits to the processing plants for the direct collection of samples. Usable samples were received from 7 honey packers and extensive data, which are included here with permission, from another.

Comb Honey Samples

A total of 41 samples of honey in the comb were received. These were crushed and separated from the wax residues by gravity straining through four layers of cheesecloth, without heat.

Table 1. Hydroxymethylfurfural (HMF) content of United States honey as received from producers

Type	No. of samples	HMF, mg/100 g Mean	s	Range
Liquid	481	0.62	0.99	0.00–13.6
Comb	41	0.27	0.26	0.03– 0.92

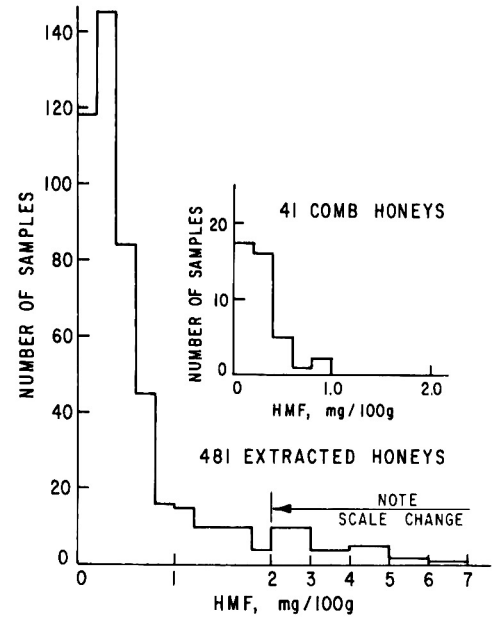

FIG. 1—Distribution of hydroxymethylfurfural content among 481 samples of extracted honey direct from producers and among 41 comb honeys.

They were prepared within 24 hr of receipt and were stored below 5°C until analyzed for HMF.

Determination of HMF

The barbituric acid-toluidine method of Winkler (2) was used, with a Stasar II spectrophotometer equipped with a flow-through cell. In this method, 5 mL 10% *p*-toluidine in 50% isopropanol containing 10% acetic acid is added to 2 mL of a 20% honey solution. Then 1 mL 0.5% barbituric acid is added, and the absorbance is read against a blank containing water instead of barbituric acid. The time-maximum absorbance occurs at 550 nm. The reaction mixture was retained in the cell until the maximum value for absorbance had been observed, the time depending on ambient temperature. The instrument was calibrated with HMF (Sigma Chemical Co., St. Louis, Missouri) which was assayed by the strength of the UV absorption maximum at 280 nm, with $\varepsilon = 16830$ as standard (13).

Results and Discussion

The HMF contents of the producer and comb honey samples are shown in Table 1. Figure 1 shows the distribution of values for both sets of samples. The comb honey samples had been exposed only to ambient temperatures. Two samples with HMF about 0.9 mg/100 g originated from warm climates (Florida and south-

Table 2. Effect of commercial processing upon hydroxymethylfurfural content of honey

Packer	Pack	Hydroxymethylfurfural, mg/100 g Before	After	Diff.	Mean diff.
A	retail	1.71	2.79	1.08	
	bulk (drum)	1.69	2.91	1.22	
	bulk (cans)	1.95	3.00	1.05	1.11
B	retail	1.48	1.36	−0.12	
	bulk	2.32	2.20	−0.12	
	bulk	5.51	7.40	1.90	
	retail	1.90	4.01	2.11	
	retail	5.58	7.60	2.02	
	bulk	5.09	8.65	3.56	1.56
C	bulk	16.7	16.7	0	
	bulk	8.72	18.5	9.78	
	bulk	2.93	6.19	3.26	
	bulk	3.85	6.21	2.36	
	bulk	2.74	4.69	1.95	3.47
D	retail	0.0	1.69	1.69	
	retail	0.0	1.64	1.64	
	retail	1.11	3.89	2.78	2.04
E	retail	1.12	1.27	0.13	
	retail	1.38	1.58	0.20	
	retail	1.12	1.12	0.00	0.11
F	retail	0.28	1.75	1.47	1.47
G	retail	0.73	2.31	1.58	
	retail	0.87	2.71	1.84	
	bulk	0.61	1.03	0.42	1.28
Average increase, all data					1.58

ern California). The extracted honey collection represents the varied heating and straining practices of the producers. Records of heating were provided; 31 producers heated their honey to 150°F or more, some holding it at high temperatures for several hours. Many heated to 120–140°F; many did not heat the honey at all. The wider range of values and the higher mean and deviation for extracted honey compared with comb honey reflects the treatment of the honey after extraction. The sample with the highest HMF content had been heated excessively (10 hr at 155°F) and stored 10 months in Florida temperatures. This degree of heat treatment is totally unnecessary and is destructive to honey flavor and aroma.

Honey is processed by heat and straining or pressure filtration to delay granulation and to eliminate yeast spores. The exact procedures used differ among packers and would be expected to have variable effects on HMF content. The results of analyses of before and after processing samples sent by cooperating packers are shown in Table 2. Retail pack is table honey in small glass containers; bulk pack usually refers to darker, lower-grade honey sold in 60 lb tins or 55 gal. steel drums for food manufacturing, but high quality, light-colored honey also is sold in bulk for this purpose. Data from 4 additional packers were not used because analytical results indicated that the same lots of honey were not followed through the processing, since after values were appreciably lower than before values. The spread in HMF increase reflects differences in individual processing practices. It seems reasonable to estimate that commercial processing increases HMF content of honey by about 2 mg/100 g honey.

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 3. Hydroxymethylfurfural content of honey during processing and packing[a]

Process	HMF, mg/100 g 1	2	3
Sampled from 55 gal. drum	0.42	0.35	0.45
After melting in hot oven	0.47	0.63	0.54
After 15 hr in settling tank	0.60	0.91	0.70
Immediately after bottling	0.58	0.94	0.84
Cased, stacked, stored 9 days	1.18	1.30	1.28
After 1 year storage	2.77	3.41	3.43
Increase from processing	0.76	0.95	0.83
Mean		0.85	

[a] Data provided by R. W. Meloy, Sioux Honey Association.

In a large, modern plant, 3 lots of honey were sampled at various stages of processing, and data for HMF content at each stage are presented in Table 3. The effectiveness of the procedure for bulk melting is indicated by the small increase in HMF content from the raw honey to post-settling sample; the increase averaged only 0.33 mg/100 g. Bottling, casing, and stack heat caused another 0.46 mg/100 g increase. The effect of one year's storage was to increase HMF levels further by an average of 255%. The average increase in HMF content (0.85 mg/100 g) resulting from the processing procedures used in this plant was smaller than any of those in Table 2, except for that of packer E, a very small operation.

Guidelines for HMF Content of Commercial Honey

The establishment of a value for the HMF content of honey beyond which a sample must

be considered possibly adulterated with invert sirup is difficult. Processing and storage of honey, even at relatively low temperatures, can add significantly to HMF concentration. Honey may be exposed to tropical ambient temperatures for months before shipment. A review of the literature (1) for this purpose indicated that a value of 20 mg HMF/100 g honey is reasonable for a guideline, based on the effect of tripling the heat exposure (processing or storage) needed to produce an HMF level of 4 mg/100 g. Data presented here are consistent with this guideline.

No sample should be condemned solely on the basis of containing 20 mg (or more) HMF/100 g honey. Other evidence of abnormal composition must also be present. For example, only 6 of 15 samples cited as adulterated (11) had an HMF content >20 mg/100 g, and 3 samples that appeared to contain no honey and consisted only of invert sirup had low values for HMF content (1.4, 1.9, 2.8 mg/100 g). In the latter 3 samples, the fructose/glucose ratio was below 1.0, and total monosaccharides were above and total disaccharides were below the honey compositional limits given in that paper. Low levels of HMF in these 3 samples indicated that the product was not an acid-invert sirup.

We concluded that knowledge of HMF content of a honey sample is informative but not conclusive of adulteration with invert sirup unless extremely high (>50 mg/100 g) values are obtained. Decisions regarding adulteration with invert sirup must be based on deviation of several compositional parameters from honey norms (11).

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Detection of Honey Adulteration By Carbohydrate Analysis

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Thirteen market samples of falsified honey containing invert sirups or conventional corn sirup and 2 labeled mixtures have been analyzed. Results are interpreted in relation to literature values for various carbohydrate constituents of honey and their vulnerability. Published data on composition of United States honey have been refined for this purpose by eliminating samples containing a major portion of honeydew, thus narrowing the compositional ranges for known honey.

Honey, as a natural sweetener, is enjoying an enhanced popularity with today's consumer. Limited availability and increased price have provided major incentives for falsification with other carbohydrate materials. In addition to the traditional adulterants such as invert sirup and conventional corn sirup (CCS), high fructose corn sirup (HFCS) has recently become available and has been used. HFCS represented a major problem until recent research provided a definitive test for its presence in honey (1).

This discussion deals with the effects of CCS and invert sirup adulteration on the carbohydrate composition of honey. The current AOAC test for admixture of CCS with honey (**31.134–31.136**, 12th Ed.) specifies separation of CCS malto-dextrins by alcohol precipitation, followed by their differentiation from fructose-containing honey oligosaccharides by paper chromatography. Aniline-diphenylamine chromogenic reagent differentiates by color between oligosaccharides with and without fructose. Replacement of the paper chromatographic step by thin layer chromatography greatly expedites the test and the improved technique has been adopted by the AOAC (2).

The only official test for the presence of added invert sirup is the qualitative resorcinol test (**31.138–31.139**, 12th ed.), which responds to hydroxymethylfurfural (HMF). This test is somewhat ambiguous, because HMF can legitimately be present in honey that has been subjected to heat or abusive storage. Quantitation provides a better understanding; this is discussed in another publication (3).

Knowledge of the carbohydrate composition of a sample is useful in judging its authenticity. Although a large body of compositional data is available for United States honey (4), its utility is somewhat limited because of the complexity of the analytical procedures heretofore needed to obtain it. Honey is such an extremely variable and complex mixture of sugars and other components (5, 6) that the relatively facile gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC) have had only limited application in studies of its composition. An HPLC method (7) for glucose, fructose, and sucrose in honey has been adopted as official first action (8), but the other carbohydrates are not well separated. Glucose and fructose may be measured in honey by GLC with lower accuracy. The complexity of honey, which has been reported to contain at least 22 di- and trisaccharides (9), severely limits attempts at quantitation. Doner *et al.* (10) described a GLC procedure in which the ratio of isomaltose to maltose is used to indicate the addition of HFCS.

Because of this complexity, the analytical system developed for an earlier survey of honey composition (2) included a separation of the sugars into monosaccharides, disaccharides, and higher sugars by charcoal column chromatography before use of conventional wet methods for quantitation within each class. The use of hypoiodite oxidation and copper reduction yielded values for glucose and fructose; sucrose was analyzed by the increase in reducing value after mild acid hydrolysis. All reducing disaccharides were reported collectively as "maltose," and higher sugars were measured as the reducing value after hydrolysis. This procedure, while entirely suitable for research purposes, is laborious and unsuited for the laboratory that performs only occasional honey analyses.

Analytical data entirely comparable to those of the earlier compositional survey have been obtained in this laboratory by simplified procedures, with only the specific glucose oxidase method used for glucose and the dry weight values of the 3 fractions from the charcoal column. This is accomplished as follows:

Monosaccharide fraction: (*1*) total monosaccharides by weight; (*2*) glucose by glucose oxidase; (*3*) fructose by difference.

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Disaccharide fraction: (1) total disaccharides by weight; (2) sucrose by invertase hydrolysis followed by glucose oxidase; (3) all other disaccharides by difference.

Higher sugar fraction: total higher sugars by weight.

In the earlier work, sucrose was measured after mild acid hydrolysis, and the value for sucrose included any melezitose present, most of which is found in the disaccharide fraction. For specific sucrose values, invertase was used. In the procedure described here, melezitose may be estimated, if required, by the difference between values obtained by the 2 hydrolytic procedures applied to the disaccharide fraction. Melezitose is a constituent of honeydew and is found occasionally in small amounts in predominately floral honeys.

Saccharimetric methods, the bases of earlier honey analyses (11), provided only estimates at best and misleading information at worst. For example, the so-called quantitative estimation procedure (**31.137**, 12th ed.) for commercial glucose (CCS) will indicate a considerable proportion of CCS when applied to a sample containing a major amount of honeydew. Several recent applications of this test to such materials have resulted in seizures that were not justified. Had the original papers been consulted and had the additional confirming tests recommended (but not included in the AOAC version) been made, these incidents would not have occurred. In a complex mixture, polarimetric analysis is of limited value, although it has been shown to quantitate quite well the glucose and fructose in the monosaccharide fraction of honey from the charcoal column (4).

Honeydew samples have been identified conventionally by polarimetry; the advisory FDA definition for honey requires that it be levorotatory (see ref. 4). Thus a significant (> 5%) amount of melezitose is a confirmatory negative test for a dextrorotatory sample that tests negative for CCS or added sucrose.

Methods for measuring the monosaccharide, disaccharide, and higher sugar content (distribution of sugars) of honey as described above have been adopted by the AOAC (8). Also adopted (8) was a method specific for sucrose which makes use of glucose oxidase to measure glucose liberated from the disaccharide fraction by invertase. The HPLC procedure for glucose, fructose, and sucrose has also been adopted (8). A collaborative test of glucose oxidase determination of glucose in the monosaccharide fraction, with fructose measured by difference, did not qualify, although it was satisfactory in the author's laboratory. The fructose value, measured by difference, was strongly affected by column performance.

A simple method that requires no sugar separation can determine true glucose in honey (12) and thus indicates adulteration if values found lie well outside the normal ranges.

Experimental

Methods

1. *Polarization.*—Constant direct polarization was determined by method **31.117**, 12th ed., with a 1 dm tube used in a Perkin-Elmer automatic polarimeter. The angular rotation values from the instrument were converted to the International Sugar Scale by multiplying by 2 (ISS values require a 2 dm tube), by 0.26; and dividing by 0.3462 (1° ISS = 0.3462 angular degrees).

2. *Distribution of sugars and determination of sucrose.* These were determined as described (8), with glucose determined by the general glucose oxidase procedure, which is used for determination of glucose in the monosaccharide fraction, and is the same as that described for the sucrose determination.

3. *Glucose and fructose.*—The monosaccharide fraction (5 mL) obtained in the method for distribution of sugars is diluted to 100 mL, and glucose is determined on 2.00 mL aliquots by the glucose oxidase procedure. For the standard glucose tubes, 2.00 mL of a glucose solution containing 100 μg glucose/mL is used.

Glucose: (mg glucose/2 mL) × 2.5 × 100/g sample on column = per cent glucose.

Fructose: Per cent monosaccharide — per cent glucose = per cent fructose.

Results and Discussion

Polarization

(Prepared with Walter F. Schmidt and Mary Rodgers, Food and Drug Administration Laboratory, Philadelphia, PA.)

The distribution of polarization values for 468 samples (Fig. 1) closely approaches a normal distribution with some tailing on the side of positive values. When the midpoint of each group is plotted, a remarkably uniform Gaussian curve results which, if idealized on the positive side, intersects the baseline at about −2°S. This implies that the empirical division at 0°S polarization between honeydew and

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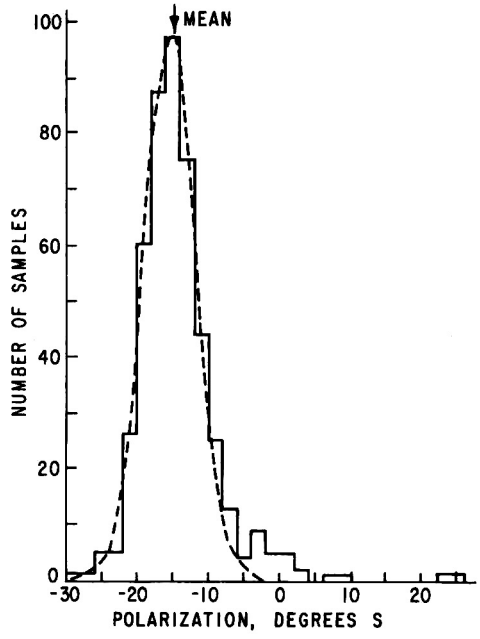

FIG. 1—Distribution of polarization (°S) for 468 honey samples, 1974–75 crop.

honey might more properly be at −2°S. In the United States this is only of academic interest, because no account is taken of the presence of honeydew in honey.

The concept that levorotatory samples are honey and dextrorotatory samples are honeydew is largely empirical; no real basis exists for such a division point except that the carbohydrates present in honeydew (melezitose and erlose) are strongly dextrorotatory. We merely attempted to use the polarization data available to indicate, by means of Gaussian symmetry, a possible lower limit of polarization for honey without appreciable honeydew content. The mean value for all 454 levorotatory samples is −14.70°S, standard deviation = 4.37°S, coefficient of variation = 29.77%.

Solids Plus Water

This value, obtained by adding the values for distribution of sugars and the water content (by refractive index, **31.112**, 12th ed.) provides insight into the adequacy of the separation and the performance of the charcoal columns. This sum should normally be between 99.0 and 101.0%. Values consistently below 99.0% indicate a defective column that should be repacked. Some minor honey constituents will not desorb, and their accumulation limits the number of re-uses of a column to about 10.

Distribution of Sugars

Based on data from the earlier survey (4), the distribution of the three groups of sugars was calculated for the 456 samples for which sugar analyses were reported.

Ranges were wide: monosaccharides, 52.56–79.95%; disaccharides, 3.29–18.16%; and higher sugars, 0.13–8.49%. Examination showed outliers on the low side of the monosaccharide distribution (7 <56%) and on the high sides of the other 2 groups: 5 with disaccharides >15% and 16 with higher sugars >4%. In several cases the same samples had outliers in more than one category.

Many of the samples used in that study had been stored frozen (0°F) since the study. Four of the 7 monosaccharide outliers, 5 of the 5 disaccharide outliers, and 7 of the 16 higher sugar outliers were available. Constant direct polarization was measured according to method **31.117**, 12th ed. All 4 of the monosaccharide outliers were honeydews (Sample 138, +4.5°; Sample 168, +14.7°; Sample 457, +3.6°; and Sample 452, +0.3°S). In the disaccharide outlier group, 2 of those named above were present; the other 3 were levorotatory. Six of the 7 available higher sugar outliers were honeydews: the 4 above, plus Sample 24, 0.00°; and Sample 459, +10.3°S.

On the basis of these results it is reasonable to assume that all of the 7 monosaccharide and the 16 higher sugar outliers were honeydew, and thus are not properly included in a study of honey variability. Elimination of these outliers from the population results in the distribution shown in Fig. 2 and summarized in Table 1.

The carbohydrate distribution of a number of market samples regarded for various reasons as possibly adulterated (Table 2) shows for 11 of the 15 samples at least one value outside the ranges in Table 1. Thus this analysis is informative and, with other data such as glucose and fructose content, can be conclusive.

Fructose Content

The mean fructose content of the 439 samples remaining after removal of honeydew outliers (Table 1) is 38.38%; the 17 outliers averaged only 33.36% fructose. The spread between extremes is decreased from the earlier value of 17.01% fructose to 13.35%; s is decreased from 2.07 to 1.77; and the corresponding coefficient

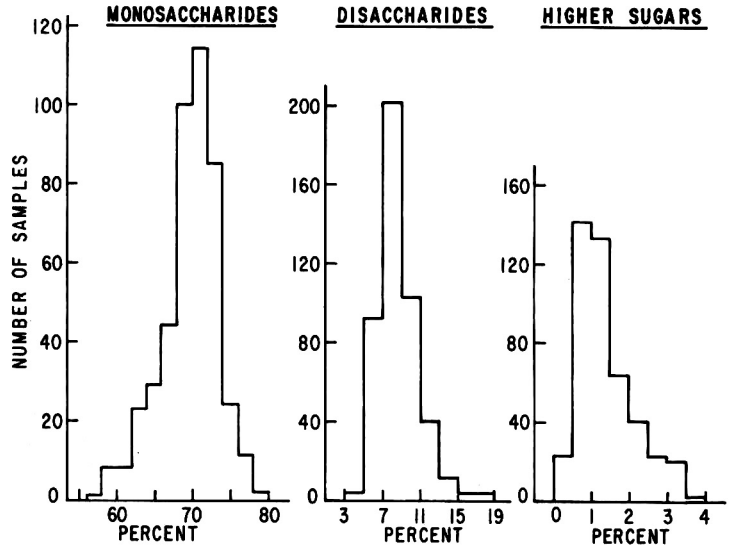

FIG. 2—Distribution of saccharides in 439 honey samples, data of 1962 (4) with honeydew outliers removed.

Table 1. Carbohydrate analysis of honey[a]

Sugar	Mean, %	s, %	CV, %	Range, %
Monosaccharides	69.71	4.13	5.92	58.00–79.95
Disaccharides	8.62	2.08	24.1	3.29–18.16
Glucose	30.31	3.04	10.03	22.89–40.75
Fructose	38.38	1.77	4.61	30.91–44.26
Fructose/glucose	1.229	0.126	10.2	0.76– 1.86
Sucrose	1.31	0.87	66.4	0.25– 7.57
Higher sugars	1.36	1.11	81.6	0.13– 3.85

[a] For 439 samples from (4), honeydew outliers removed (see text).

of variation is decreased from 5.42 to 4.61%. The coefficient of variation for fructose content was the smallest known for a honey constituent or property until that for the $^{12}C/^{13}C$ isotope ratio was determined to be 3.73% (1). Three of the samples in Table 2 are below this lower limit for fructose.

Glucose Content

Removal of the 17 honeydews from the sample population decreased the average glucose content from 31.28 to 30.31% and reduced the range between limits from 18.97% glucose[1] to 17.86, but had little effect on s and the coefficient of variation. Three of the samples in Table 2 exceed the upper limit.

Thirteen of the 456 samples analyzed earlier (4) contained more than 36.0% glucose. Two were described as from the athel tree (*Tamarix aphylla*), 6 from cotton (*Gossypium hirsutum*), 1 each from dandelion (*Taraxacum officinale*), heartsease (Polygonum spp.), blue curls (*Trichostema lanceolatum*), and manzanita (Arctostaphylos spp.), and 1 was an autumn desert blend. Four of these exceeded 38% glucose: 1 each from athel tree, cotton, blue curls, and manzanita. Rapeseed honey, known to granulate rapidly, would also be expected to be high in glucose. Because no samples from this source were included in the 1962 study, 10 samples of this type of honey were obtained from a Canadian source and analyzed for glucose by the direct glucose oxidase procedure (12). The average was 36.27%, range 34.54–37.15%, s = 0.75. A glucose content >38% in a sample may be considered as contributory evidence of its falsification in the absence of pollen from the 4 sources listed above.

Fructose/Glucose Ratio

The dispersion of the fructose and glucose values for the samples included in Table 1 is shown in Fig. 3, and the distribution of fructose/glucose values is shown in Fig. 4. Two of the 439 samples have fructose/glucose <1.00. One, preserved since the earlier work, was reanalyzed, verifying the value. The other was from blue curls, a honey earlier reported (13) to have fructose/glucose <1. Two samples of

[1] The value of 22.03% in Table 1 (4) given for the lowest glucose content is in error; Sample 168 had 21.78%.

Table 2. Carbohydrates in questioned samples

No.	Monosaccharides			F/G	Disaccharides		Higher sugars total, %	HMF, mg/100 g	Probable additive
	Total, %	Fructose, %	Glucose, %		Total, %	Sucrose, %			
1	69.1	34.6	34.5	1.00	13.8	9.7	1.65	97.5	invert sirup
2	48.7	23.2	25.5	0.91	9.4		17.6	16.7	corn sirup
3	81.2	39.9	41.3	0.97	1.8	0.3	0.3	1.4	invert sirup[a]
4	46.5	21.4	25.1	0.85	17.3	2.6	13.5	17.5	corn sirup
5	80.7	40.5	40.2	1.01	1.9		0.4		invert sirup[a]
6	63.6	33.4	30.2	1.11	16.0	13.2	6.0	46.3	invert sirup
7	75.0	37.1	37.9	0.98	7.5	0.7	1.3	45.8	invert sirup[a]
8	68.8	30.4	38.4	0.79	8.0	4.6	5.5	87.5	invert sirup
9	76.9	38.8	38.1	1.02	5.2		0.9	4.3	invert sirup
10	69.7	34.2	35.5	0.96	11.5	4.7	1.5	25.6	invert sirup
11	60.8	36.0	24.8	1.45	18.3	12.6	2.4	4.9	
12	76.9	33.4	43.5	0.77	2.0	0.3	0.3	1.9	invert sirup[b]
13	71.4	33.7	37.7	0.89	6.5		1.4	36.3	invert sirup[a]
14	84.2	37.7	26.0	1.45	17.6	13.1	2.1	14.0	
15	80.5	39.3	41.2	0.95	2.7	0.4	0.5	2.8	invert sirup[a]

[a] Apparently contained no honey.
[b] Honey substitute, labeled as containing invert sirup, not a questioned sample.

blue curls honey from California (1975 crop) were analyzed by the method described above. The reported preponderance of glucose over fructose characteristic of this honey type is confirmed (Table 3). The effect on fructose/glucose of adding invert sirup, which normally has fructose/glucose <1, or of adding CCS, is also apparent in Table 2.

Sucrose

(Prepared with A. P. Hoban, Eastern Regional Research Center, Philadelphia, PA.) The sucrose content of honey is normally rather low; White *et al.* (4) gave for 490 samples a mean of 1.31%, $s = 0.95$, range 0.25–7.57%. This included 7 samples >5.0%; one of these was eliminated when outliers were removed. Of the

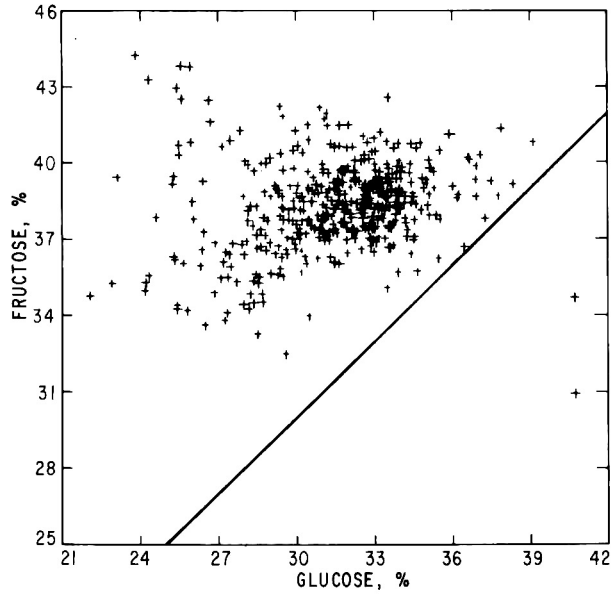

FIG. 3—Fructose and glucose relationship for 439 honey samples. Line indicates F/G = 1.

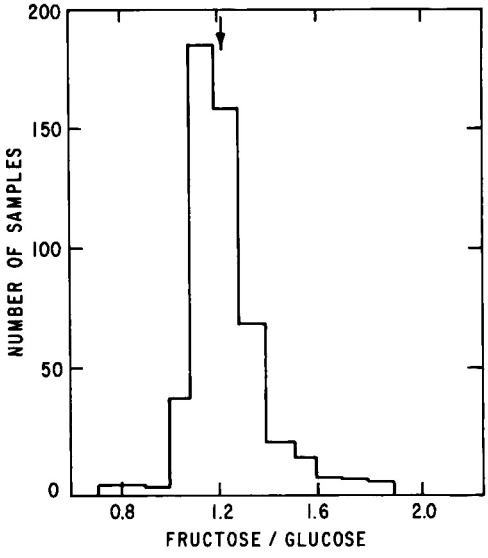

FIG. 4—Distribution of fructose/glucose ratio in 439 honey samples, data of 1962 (4) with honeydew outliers removed.

6 remaining, 3 were alfalfa or alfalfa-sweet clover, 1 citrus, 1 star thistle (*Centauria solstitalis*), and 1 hairy vetch (*Vicia villosa*).

A collection of 481 honey samples of the 1974–1975 crops, certified authentic by their producers, had been obtained for a project to develop means to detect the addition to honey of high fructose corn sirup. These were analyzed for sucrose by the procedure described elsewhere (8). The distribution of values is shown in Fig. 5. It resembles that in the earlier work (4), but a much greater proportion is found in the 0–0.5% group, possibly because of the greater sensitivity of the method used. The mean was 1.21%, $s = 1.30$, and range 0.03–9.74%. Thirteen of these samples exceeded 5%; only one (a California orange honey) was higher than the 8% cited in the FDA advisory definition. Floral types of these 13 samples were 1 citrus, 1 locust (Robinia spp.), 7 alfalfa alone or mixed with sweet clover, 1 macademia nut, 1 unknown.

Table 3. Analysis of honey from blue curls (*Trichostema lanceolatum*)

Sample	Fructose, %	Glucose, %	Ratio, F/G
149 (1962)	30.91	40.75	0.76
402 (1975)	31.91	38.15	0.84
428 (1975)	34.04	37.52	0.91

The data from these 2 analytical studies showed that 19 of 919 samples of United States honey had sucrose contents greater than 5%; citrus (4 samples), alfalfa, and alfalfa-sweet clover (10 samples) were the types most frequently high in sucrose. Most of these types cited are known to be deficient in natural invertase and are slow to ripen. The sucrose content of all natural unheated United States honeys will decrease to <5% in a few months at room temperature; a high sucrose adulterated product is more stable. The time may be reduced to a few weeks by diluting the sample (if necessary) to 18.6% moisture and holding it at 37°C.

The sucrose contents of 41 additional samples received in the comb also are shown in Fig. 5. For these samples, obtained directly from the hive and refrigerated upon receipt, the mean sucrose content is 2.78%, appreciably higher than that for the extracted samples. This may be ascribed to their having less storage time during which to ripen by action of the natural invertase.

The advisory FDA definition of honey allows a maximum of 8% sucrose. The Codex Alimentarius limit (14) for apparent sucrose (increase

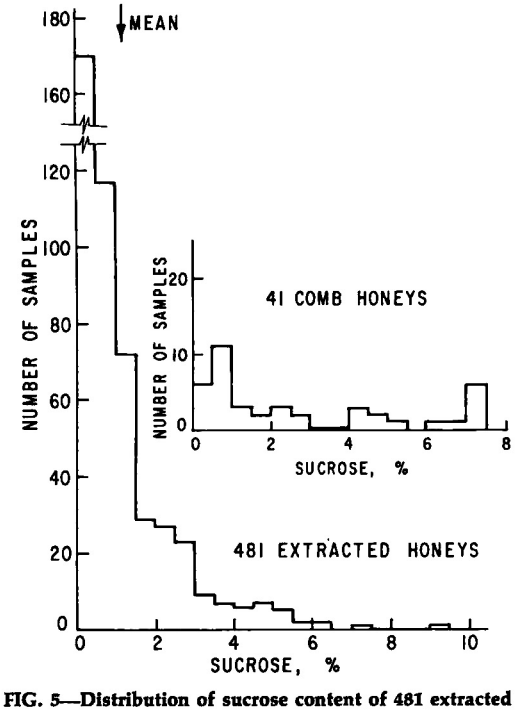

FIG. 5—Distribution of sucrose content of 481 extracted honey samples and 41 comb honey samples, crop years 1974–75.

in reducing sugars after mild acid hydrolysis) is 5%; but 10% is permitted for specified floral types known frequently to be high in sucrose (Banksia, lavender, Robinia). The high sucrose values for several samples in Table 2 is contributory evidence of their falsification.

Other Disaccharides

The analytical procedures described herein will not permit quantitation of other individual disaccharides. Although several GLC procedures for honey analysis have been reported (15–18), rigorous evidence of complete separation of the reported disaccharides has not been forthcoming. Doner *et al.* (10) described a GLC procedure for measuring isomaltose/maltose ratio for use in selecting samples for isotope ratio analysis for HFCS; this procedure can provide information useful for distinguishing synthetic mixtures sold as honey, because all honeys examined contained these sugars. The total absence of maltose and isomaltose from several of the samples listed in Table 2 (Nos. 5, 12, 15) when analyzed by the GLC procedure confirmed that they contained no honey.

Higher Sugars

The distribution of higher sugars found in the earlier study, after the correction for honeydew noted above, is shown in Fig. 2 and Table 1 for levorotatory honeys. The finding of more than 4.0% higher sugars in a levorotatory sample may be considered contributory evidence of falsification (Table 2).

Hydroxymethylfurfural

This compound is present in nearly all honey; its content depends on the storage and heating history of the specific sample. The use of HMF analysis for detection of adulteration is discussed in another paper, as is the analysis of samples from the 1974–75 crops (3).

Other Carbohydrates

The minor di- and trisaccharides of honey are considered to originate largely from the transglucosylation accompanying the inversion of nectar sucrose by honeybee invertase and from acid reversion.

Saccharides produced by glucose transfer during honey ripening are known to differ from those resulting from the fructose transfer characteristic of yeast invertase inversion of sucrose (5). The sugars obtained from Sample 3 in Table 2 were examined by paper chromatography. Migrations on paper of these sugars was typical of those reported by Bacon and Edelman (19) for yeast transfructosylation intermediates, differing from those known for the transglucosylation typical of honey invertase (20). This, together with low HMF levels and other considerations, is consistent with the product's identity as an invert sirup produced by enzyme inversion.

The wide variability in honey composition requires for demonstration of adulteration that a number of parameters be outside expected norms. In addition to the carbohydrate components discussed here, such factors as acidity (free, lactone, and total), ash, proline (21), and protein (22) may be included.

Carbohydrate analysis as described here is of little or no value in detecting the addition of HFCS to honey; the material sufficiently resembles honey in its major constituents and is so purified that its addition does not change composition sufficiently for reliable detection. The use of $^{13}C/^{12}C$ ratio (1) does provide an absolute method for this purpose. In addition, Kushnir (23) has developed a thin layer chromatographic procedure that, when applied to a suitably prepared concentrate of trace higher sugars, will detect the addition of 5% or more HFCS to honey. This has been successfully tested collaboratively (2). A suggested order of procedure is shown below for the analyses of a questioned sample. A wider and more definitive set of analytical guidelines is in preparation which will differentiate in more detail between genuine and adulterated honey, based on data from this and other sources.

Detection of Honey Adulteration: Recommended Order of Procedure

Perform test A and/or B below:

A. *Determine* $\delta^{13}C$ *(1):* Values less negative than −21.5‰ are conclusive for adulteration with corn or cane sirups. Values between −21.5‰ and −23.4‰ are inconclusive and require testing by the TLC method. Values more negative than −23.4‰ characterize pure honey.

B. *Apply TLC test (23):* Positive test demonstrates presence of about 5% or more of high fructose or conventional corn sirups, which can be differentiated by determining distribution of sugars, glucose content, and fructose/glucose

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

ratio. If TLC test is negative, carry out HMF test.

Determine HMF content (3): The following conclusions can be drawn from the amount of HMF found in the sample:

20 mg/100 g or more: Adulteration with invert sirup is strongly implied. Confirm with distribution of sugars, glucose, sucrose, and fructose contents.

10–20 mg/100 g: Sample may be heat- or storage-abused honey. Carbohydrate analysis should differentiate between such honey and adulterated honey.

<10 mg/100 g: Determine glucose content. If >40%, sample is not genuine; if between 38–40%, absence of specific pollens indicates adulteration; if <38%, sample is probably genuine.

Acknowledgments

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FOODS

Spectrofluorometric Determination and Thin Layer Chromatographic Identification of Serotonin in Foods

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A method is described for determining serotonin in foods, based on alkaline butanol–sand column elution followed by spectrofluorometric and spectrofluorometric determination with thin layer chromatographic confirmation. The method has been applied to fresh bananas, banana-based baby foods, and fresh and canned tomatoes. The average recovery was 91%. The amounts of serontonin found were 10–30 ppm in bananas, 0.1–1.9 ppm in banana-based baby foods, 4.4–5.6 ppm in fresh tomatoes, and 2.8–5.6 ppm in canned tomatoes.

Serotonin is found not only in animal and vegetable tissues but also in various foods. It is of toxicological interest as a biogenic amine and also because it can interact with monoamine oxidase inhibiting drugs. Its presence in foods, therefore, may have undesirable effects; however, its presence may also be beneficial (1).

Serotonin has been studied in a number of biological media, and those methods have been adapted for its determination in foods (2–7). However, no method specifically designed for its determination in foods has been described in the literature. Such a method is described in this paper.

Serotonin is extracted from the sample by alkaline butanol–sand column elution, transferred to 0.1*N* HCl, determined by spectrofluorometry, and verified by spectrofluorometry and TLC. This method was applied to fresh bananas, banana-based baby foods, and fresh and canned tomatoes.

METHOD

Apparatus

(a) *Spectrofluorometer.*—Aminco SPF 125.

(b) *Rotovapor.*—Buchi R.A. with W-240K bath.

(c) *TLC plates.*—For 5 plates, 20 × 20 cm, homogenize 35 g silica gel G (type 60; Merck) and 70 mL water. Activate plates at 110°C for 30 min before use.

(d) *Glass columns.*—500 × 30 mm, with No. 0 filter in bottom, and stopcock (Afora).

Reagents

(a) *Serotonin-creatinine sulfate monohydrate.*—(Merck). This complex was used throughout study, with concentrations expressed as function of pure serotonin.

(b) *Alkaline butanol.*—Mix a few drops (ca 1 mL) of saturated aqueous solution of NaOH with analytical reagent grade *n*-butanol to pH 9–10.

(c) *p-Dimethylaminobenzaldehyde.* — Dissolve 20 g *p*-dimethylaminobenzaldehyde (Merck) in 75 mL methanol and 25 mL HCl.

(d) *Ninhydrin.*—Dissolve 0.2 g ninhydrin (Merck) in 100 mL ethanol.

(e) *o-Phthalaldehyde (OPT).*—Dissolve 0.5 g OPT (Fluka) in 100 mL ethanol.

Calibration Curve

Prepare 1 mg/mL solution of serotonin in 0.1*N* HCl, and from this, prepare solutions containing 15, 75, 100, 150, 225, 450 μg/120 mL 0.1*N* HCl. Prepare fresh every day. Read excitation wavelength at 295 nm and emission wavelength at 340 nm against 0.1*N* HCl blank. Obtain new calibration curve for each series of determinations.

Extraction and Separation

Weigh 7.5 g bananas, or 15 g foods containing less serotonin (i.e., tomatoes and baby foods). Mechanically homogenize bananas and tomatoes; baby foods may be shaken. Place homogenized sample in 200 mL beaker, add fine sand, and shake until mixture loses its viscous consistency. Add 50 mL alkaline butanol and let stand 0.5 hr, shaking occasionally. Pack this mixture into glass column which is shielded with black paper (serotonin is sensitive to light). Wash beaker with 150 mL alkaline butanol and add wash to column.

Extraction proceeds as drop-by-drop elution of butanol from column. Adjust elution to last ca 1 hr. Add 10 mL petroleum ether to butanol eluate. Extract with six 20 mL portions of 0.1*N* HCl to obtain a final volume 120 mL.

Spectrofluorometry

Quantitative analysis.—Read fluorescence values of HCl solution at maximum excitation wave-

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Table 1. Reproducibility of described method for 9 serotonin determinations on a single batch of baby food

Sample	F^a	$F - \bar{F}$	$(F - \bar{F})^2$
1	39	−1.22	1.48
2	41	0.78	0.60
3	41	0.78	0.60
4	38	−2.22	4.92
5	40	−0.22	0.04
6	42	1.78	3.16
7	42	1.78	3.16
8	38	−2.22	4.92
9	41	0.78	0.60

Std dev., 1.56
Rel. std dev., %, 3.87

[a] Units of fluorescence, scale 0–100.

length 295 nm and maximum emission wavelength 340 nm.

Qualitative analysis.—Compare excitation and emission spectra of HCl solution to ensure that they coincide with those of pure serotonin.

Thin Layer Chromatography

To eliminate interference in TLC procedure, clarify extracts with lead acetate:

To 120 mL 0.1*N* HCl solution, add 15 mL saturated aqueous solution of lead acetate, and filter. Add solid solution oxalate to filtrate until precipitation ceases, and then repeat filtration procedure. Concentrate filtrate to 1 mL in Rotovapor at ≤40°C. Add 1 drop of concentrated HCl, and use resulting filtrate or decanted mixture for TLC. On one TLC plate, spot standard serotonin, sample extract, and serotonin standard and sample superimposed. Use either developing solvent: ethanol–ammonia 25% (4+1) (8), or chloroform–ethanol–acetic acid (70+20+40).

Spray with OPT; serotonin gives a characteristic brown-yellow fluorescence at 360 nm.

Results and Discussion

Blank apricot and apple baby food was selected on the basis of the lack of the characteristic fluorescent properties of the amine in the serotonin region. Then 10 ppm serotonin was added, and 9 replicate determinations were carried out by the described method (Table 1). The relative standard deviation (RSD) was 3.87%.

Different amounts of serotonin, 1, 5, 7.5, 10, and 15 ppm, were added to samples of apricot and apple baby foods; each level was assayed in triplicate (Table 2). Average recovery of the 15 assays was 91%; the RSD was 3.79%.

We also applied the described method to various samples of food acquired commercially. Results are shown in Table 3.

Table 2. Recovery (%) of serotinin added to different samples of same baby food

Added, ppm	F,[a] std	F, sample	Rec., %	Av. rec., %
1.0	9.0	7.2, 9.0, 9.0	80, 100, 100	93
5.0	22.0	19.0, 18.0, 20.5	86, 81, 93	86
7.5	31.0	26.0, 27.0, 26.0	83, 87, 83	85
10.0	40.0	38.0, 41.0, 41.0	95, 102, 102	99
15.0	54.0	54.0, 50.0, 49.0	100, 92, 90	94
Av. rec.				91
Rel. std dev., %				3.79

[a] Units of fluorescence, scale 0–100.

To study the effects of possible interferences, the following chemicals were tested: DL-5-hydroxytryptophan (Ega-Chemie), tryptamine monohydrochloride (Merck), D-tryptophan (Sigma), tyramine monohydrochloride (Merck), L-adrenaline (Merck), and creatinine (Merck). Figure 1 shows the corresponding spectra.

It appears that 5-hydroxytryptophan interferes. However, this compound is not present in the final extracts used for determination and identification, which agrees with the results found by other authors (2, 3, 4, 7). To confirm this, we applied the proposed method to samples without 5-hydroxytryptophan and samples to which 20 ppm 5-hydroxytryptophan had been added. The recovery was very low (max. 20%), so even if 5-hydroxytryptophan were present, the amount extracted would be too little to interfere. In addition, the intensity of fluorescence of 5-hydroxytryptophan, under the same conditions, is much less than that of serotonin. We have also determined that the addition of lead acetate, under our experimental

Table 3. Serotonin content (ppm) of commercial samples

Banana	Serotonin	Banana-based baby food	Serotonin	Tomato[a]	Serotonin
1	10.0	1	1.9	1	4.4
2	18.0	2	1.6	2	4.7
3	20.6	3	1.9	3	4.4
4	16.8	4	1.0	4	5.6
5	16.2	5	1.0	5	5.3
6	17.4	6	1.9		
7	19.4	7	—		
8	17.4	8	0.1	1C	5.6
9	19.9	9	0.7	2C	4.4
10	22.6			3C	2.8
11	26.6			4C	4.9
12	30.0				

[a] C = canned.

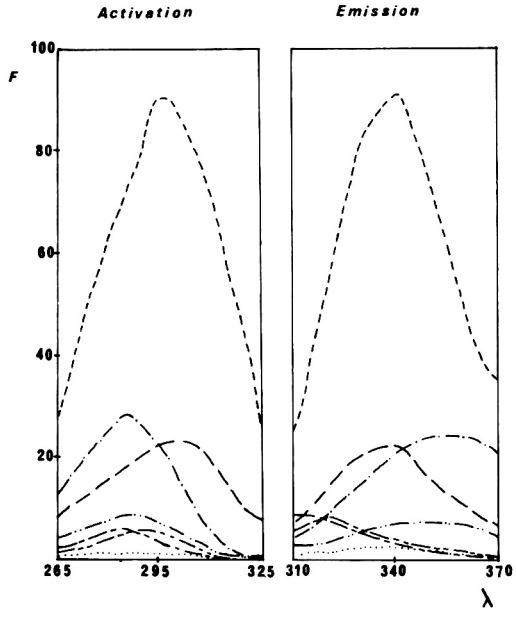

FIG. 1–Fluorescence spectra of serotonin and possible interferences: ----- serotonin; ——— 5-hydroxytryptophan; —.—.—. tryptamine; —..—..—.. tryptophan; —— —— tyramine; ——— ——— adrenaline ..... creatinine. Concentration: 0.3 mg/120 mL 0.1*N* HCl.

conditions, does not eliminate 5-hydroxytryptophan, which confirms its absence in the samples analyzed. Tryptamine and the other possible interferences also showed less intense fluorescence. The excitation and emission spectra for all our samples matched those of serotonin exactly.

When the thin layer chromatograms were treated with other reagents (ninhydrin, *p*-dimethylaminobenzaldehyde), spots other than serotonin became visible. OPT reveals only serotonin and creatinine; the latter was observed in all samples analyzed.

Some of the samples in which serotonin was not detected by fluorometry gave weak spots for serotonin on thin layer chromatograms. Either serotonin is present in very low amounts or some substance very similar to serotonin is present. This discrepancy only occurred for prepared foods (in our case, baby foods), not in natural products. We thought that serotonin might have undergone some change or modification as a result of food treatment. We tested serotonin dissolved in pH 4.6 buffer (9) and subjected to temperatures of 100°C. The treated serotonin lost its fluorescence, but could still be detected chromatographically. Thus, quantitative results for heat-treated products might be low. It would be interesting to investigate the exact nature of the modification or reaction that affects the serotonin under these conditions and whether it affects the biological activity.

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OILS AND FATS

Lipid Extraction Procedure for Some Food Products Containing Surfactants

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Total lipids were extracted from food products by heating the sample under total reflux for 15 hr with a mixture of chloroform–methanol (70+30) in the ratio 6.67 mL solvent/g sample. The solution was filtered and evaporated, and the lipids were dissolved in petroleum ether, filtered, and evaporated to yield the crude lipids and surfactants. The purified lipids are suitable for butterfat determination directly, or are readily separated from the surfactants by alumina chromatography. The method is particularly suited to the milk products studied, which produced troublesome emulsions if aqueous treatments were used. The method also allows ready extraction of total fats from drained feta cheeses, whereas Soxhlet extraction with petroleum ether sometimes fails. The effects of reflux time and varying ratios of chloroform to methanol were studied.

For purposes of classification within the Tariff Schedules of the United States and/or compliance with quota limitations, the Customs Service frequently needs butterfat determinations. The butterfat analyses are of 2 general types: (a) those which determine the total fatty material in the foodstuff, and then the per cent butterfat; (b) those which ascertain the animal origin (cow, goat, etc.) of the butterfat. In the former case, all the fats must be extracted or the analysis may result in a misclassification. For the butterfat assay, the Reichert-Meissl, Polenske, and Kirshner (RMPK) values (1, 2) are determined on 5 g samples of fat. Recently, we have encountered a series of food products, generally the flavored powdered-milk, breakfast food variety, which do not allow ready extraction of all the fats present.

The AOAC methods of extraction previously used included **16.181** for fat in dried milk (3); **14.132** (dilute HCl hydrolysis for macaroni, egg noodles, and similar products (4)); **16.232(b)** (dilute H_2SO_4 digestion for cheese (5)); and Soxhlet extraction with petroleum ether (bp 30–60°C). The Soxhlet extraction gave low results in several feta cheeses. The three AOAC methods failed in the organic solvent extraction step, which follows the initial base and/or acid treatment of the sample, because of the formation of stable emulsions. Efforts which involved changing the extraction solvent from the usual 1 + 1 petroleum ether–ethyl ether to ether, petroleum ether, chloroform, or mixtures of the three were not successful, nor was centrifugation or the addition of emulsion foam breakers (e.g., Dow Corning Antifoam A spray).

To circumvent the problem of emulsions, we studied single phase extraction with complete exclusion of water.

METHOD

Apparatus

(a) *Gas-liquid chromatograph.*—Varian Aerograph Model 1800 equipped with flame ionization detector. Operating conditions: detector, 300°C; injection port, 275°C; temperature program: 4 min at 60°C, 6°/min to 204°C, hold 8 min, 8°/min to 280°C; helium carrier gas flow, 30 mL/min; hydrogen 30 mL/min; air 300 mL/min; 1.8 m × 2 mm id glass column packed with 10% Superox 4 on 80–100 mesh Chromosorb W (NAW) (Alltech Associates, 606A Union St, Kennett Square, PA 19348).

(b) *Integrator.*—Hewlett-Packard Model 3352B Laboratory Data System.

(c) *Mass spectrometer.* — Hitachi-Perkin-Elmer RMU-6L interfaced to Perkin-Elmer 3920 gas chromatograph with flame ionization detector and the integrator. Operating conditions: detector, 275°C, injection port, 250°C; temperature program: 4 min at 60°C, 4°/min to 220°C; helium carrier gas flow 30 mL/min; hydrogen 40 mL/min; air 550 mL/min; 1.8 m × 2 mm id glass column packed with 10% Carbowax 20M on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA 16823).

(d) *Infrared spectrophotometer.*—Perkin-Elmer Model 457 grating infrared spectrophotometer. Sample spectra were recorded between KBr discs as neat film or Nujol or Fluorolube mulls.

(e) *Chromatographic column.*—Insert pledget of glass wool at lower end of 290 × 32 mm glass column. Fill column ⅔ full with $CHCl_3$ and add ca 200 g alumina slurried in $CHCl_3$; pack with

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gentle tapping. Top column with 2 cm acid-washed sand.

(f) *Proton magnetic resonance spectrometer.*—Varian Associates, Model T-60; use solutions in $CDCl_3$ with TMS internal standard.

Reagents

All solvents should be ACS grade.

(a) *Tetramethylammonium hydroxide (TMAH).*—(Aldrich Chemical Co.). 20% in methanol.

(b) *Alumina, adsorption.*—80–200 mesh (Fisher Scientific Co.).

(c) *Coconut acid amides.*—Courtesy of Scher Chemicals, Inc., Industrial West, Clifton, NJ 07012).

(d) *Lecithin.*—Kecon G. J., powder (Lucas Meyer, West Germany).

Extraction Procedures

Place sufficient finely divided sample to yield 5 g fat (e.g., 90 g Cocolac) into 1 L flat-bottom flask equipped with magnetic stirrer and fitted with water condenser-drying tube ($CaCl_2$). Add 6.67 mL solvent/g sample, from a mixture of 180 mL anhydrous methanol and 420 mL $CHCl_3$. Thoroughly stir and heat mixture under total reflux for 7 hr. Suction-filter cooled suspension, and rinse filter cake with 400 mL $CHCl_3$. Evaporate filtrate under stream of air on steam bath. Intimately mix crude semisolid thus obtained with five 100 mL portions of petroleum ether (bp 30–60°C), and filter each portion by gravity through 11.0 cm Whatman No. 42 paper. Rinse filter with three 50 mL portions of petroleum ether. Evaporate combined petroleum ether filtrates on steam bath and then weigh crude extracted fats.

Treat filter cake as in **16.232(b)** (5), with external cooling during addition of H_2SO_4. Cover deep brown suspension and let stand overnight. Extract residual fats from H_2SO_4 solution with four 250 mL portions of ether-petroleum ether (1+1), wash with three 400 mL portions of brine, dry over Na_2SO_4, evaporate solvents on steam bath, and weigh residual fat.

Drain and pat dry the sample of feta cheese on clean paper towels; grate to coarse pieces. Place sufficient grated sample (e.g., 50 g) in thimble for Soxhlet extraction (Whatman Double Thickness 43 × 123 mm). Place 30 mL petroleum ether (bp 30–60°C) into weighed 500 mL flask and let Soxhlet extraction proceed 6–7 hr. Evaporate solvent on steam bath, and heat fat sample in forced draft oven 20 min at 105°C.

Chromatography and Identification

Dissolve and transfer extracted fats (5.7 g from Cocolac sample) in minimum $CHCl_3$ and place at top of 200 g slurry-packed alumina column (2.9 × 32 cm). Elute with 100 mL hexane; evaporate eluate in weighed glass dish on steam bath. If >4–6 mg material is obtained, continue eluting with 100 mL portions of hexane until fractions <4–6 mg are obtained. Then elute with 100 mL portions of hexane–chloroform mixtures containing increasing amounts of chloroform (e.g., 20, 50, 100% chloroform) until fractions <4–6 mg are obtained. Then elute with 100 mL portions of chloroform–95% ethanol mixtures containing increasing amounts of ethanol (e.g., 20, 50, 100% of 95% ethanol) until total weight recovered does not increase significantly. If significant amounts of material are still unrecovered, boil alumina column packing in chloroform–methanol (70+30), filter solvent, and evaporate on steam bath. This step may have to be repeated.

Discard fractions containing <6 mg material. Record IR spectra of remaining fractions, and combine those that give similar spectra. Characterize fractions which are not fats by chemical tests, nuclear magnetic resonance, gas chromatography, gas chromatography–mass spectrometry, as needed.

Results and Discussion

The chloroform–methanol extraction worked in all the food products studied and was clearly superior to alternative procedures compared (Table 1). The proposed procedure generally affords a higher yield of lipid material, but the extent of this improvement varies considerably

Table 1. Comparison with alternative procedures of total lipid material extracted by $CHCl_3$-methanol

Sample	Per cent lipid material obtained: $CHCl_3$-methanol[a]	Alternative procedure	Manufacturer's claim
Cocolac (Canada)	6.38	2.4; 2.4; 2.6[b] 2.7; 2.9[c] 6.35[d] 6.79[e] 2.4[f] 5.84[g]	
Milo (Jamaica)	10.23; 10.76	4.60; 4.70[b]	10.6
Milo (Australia)	9.94; 10.68; 10.02	2.3; 7.4[d]	10.0
Ovaltine (England)	2.64	2.5[d] 0.26[b]	
Chlorella Mill (Japan)	17.04	16.41[b]	
Klim (USA)	24.94; 24.55	3.61[b]	min. 26.0

[a] $CHCl_3$-methanol (70+30), 7 hr reflux.
[b] Soxhlet extraction with petroleum ether, bp 30–60°C, 5–7 hr.
[c] Centrifugation with petroleum ether, bp 30–60°C.
[d] AOAC **16.055**, Roese-Gottlieb method.
[e] AOAC **16.232(b)**, reported by Fitelson Labs.
[f] Soxhlet extraction with ethyl ether, 5–7 hr.
[g] Soxhlet extraction with acetone, 15 hr.

Table 2. Effect of reflux time on lipid material extracted by $CHCl_3$-methanol

Sample	Reflux time, hr	Per cent lipids, extracted[a]	Per cent lipids, residual[b]	Per cent lipids, total[c]
Cocolac	7	6.38	0.03	6.41
	14	6.62	0.05	6.67
	15	6.78	0.02	6.80
Ovaltine	7	2.64	0.24	2.86
	14	2.82	0.03	2.85
Milo (Jamaica)	7	10.23	0.10	10.33
	14	10.76	0.01	10.77
Klim	7	24.94	0.04	24.98
	15	25.15	0.03	25.18

[a] Lipids extracted by $CHCl_3$-methanol (70+30).
[b] Lipids recovered from the extracted residue by dilute H_2SO_4 digestion: AOAC **16.232**(b).
[c] Sum of procedures given in *a* and *b*.

from product to product in an unpredictable manner.

The effect of reflux time was studied for many of the samples (Table 2). Generally, there is a small increase in the amounts of extracted fats obtained as the reflux time is increased from 7 to 15 hr. Both Ovaltine and Milo show this effect via a marked decrease in residual lipids after 15 hr of reflux. Again, the efficiency of the extraction depends on the sample and cannot be predicted. We suggest that 15 hr be taken as a minimum reflux period, and that **16.232(b)** (5) be used to ascertain if appreciable lipid material was not extracted.

The effect of varying the ratio of $CHCl_3$ to methanol was studied on one sample (Table 3). The use of $CHCl_3$–methanol (70+30) as solvent appears to be desirable based on the criterion of the least residual lipid material obtained by **16.232(b)** (5). For most of this work, the 70+30 ratio was used. The maximum extraction (or minimum residue) may shift from sample to sample. Routine use of 15 hr extraction and 70+30 solvent ratio is expected to succeed in nearly all cases that require this procedure.

The use of chloroform–methanol–water (50+100+40) as solvent in a blender has been described for other materials (6). Our experience suggests that with these samples water is to be scrupulously avoided lest stable emulsions ensue. Two runs were performed with Ovaltine unprotected by $CaCl_2$ desiccant on very humid days. (Ovaltine appeared to be the most hydroscopic of this group of samples.) Sufficient water was absorbed by the refluxing solvent to convert the stirred Ovaltine to a syrupy mass that was resistant to efficient stirring and filtering. The procedure was repeated, and again the Ovaltine could not be extracted successfully.

The chief differences from the original suggestion of Folch-Pi *et al.* (7) are that the $CHCl_3$-methanol extract is not washed with 0.2 volumes of water (or salt solution), and that the solvents are refluxed. In agreement with some current reports (8, 9), the $CHCl_3$-methanol lipid extraction is superior to other methods. For samples that tenaciously retain lipid material, the proposed refluxing with $CHCl_3$-methanol should be efficacious. As noted in Table 2, the amount of total recovered lipidic material increases on prolonged refluxing. It is not to be expected that equally effective extraction can be obtained for the samples studied by centrifugation at room temperature after blending, e.g., as in refs. 8 and 9.

Feta cheese, which is packed in water, has sometimes presented problems of very low yields of fat when Soxhlet-extracted with petroleum ether. Lipids are completely extracted from such samples, after careful draining and crumbling, in a few hours with this procedure. The lipids thus obtained are determined by GLC: the ratio of C_{10}/C_{12} and C_{12}/C_{14} methyl esters allows the animal origin to be determined (10).

The proposed procedure deliberately extracts all the fats and surfactants together. Then a butterfat analysis may be run on the lipidic

Table 3. Effect of varying ratio of $CHCl_3$ to methanol on lipid extraction from Milo

Solvent ratio, v/v	Per cent lipids, extracted	Per cent lipids, residual[a]	Per cent lipids, total[b]
80+20	9.72	0.08	9.80
	10.04	0.10	10.14
70+30	9.94	0.04	9.98
	10.68	0.04	10.72
	10.02	0.04	10.06
60+40	10.60	0.06	10.66
	10.61	0.05	10.66
50+50	10.95	0.06	11.01
	10.51	0.05	10.56

[a] Lipid material recovered from extracted residue by dilute H_2SO_4: AOAC **16.232(b)**, and solvent extraction.
[b] As obtained from both procedures.

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Table 4. Column chromatographic separation of $CHCl_3$-methanol extracted lipid material

Fraction No.	Vol., mL comp. solvent	Fraction[a] wt, g	Identity	Identification procedure
2–4	300 $CHCl_3$	4.9421	butter and cocoa fats	IR (11)
8–10	100 $CHCl_3$ + EtOH (4+1)	0.1384	lecithin	IR; pos. N, P (12), dye transfer tests (13)
	100 $CHCl_3$ + EtOH (1+1) 450 EtOH			
11	1800 EtOH	0.0686	lecithin and free fatty acids	IR
12	8 hr Soxhlet extraction $CHCl_3$ + MeOH (70+30)	0.3062	free fatty acids and Na salts thereof	IR; pos. N, neg. P; GLC; GLC/MS (15)
13	as above	0.1540	as above	as above

[a] The weight recovered is 5.6093 g (97.75%) of which the fats are 88.1%; lecithin is minimum 2.47%; fatty acids and sodium salts thereof are minimum 8.20%. The Reichert-Meissl, Polenske, and Kirschner butterfat determinations on another sample of fats extracted by this method are 80.4% of the total lipid material. Fractions 1, 5, 6, and 7 were discarded because they contained less than 6 mg material.

material. The butterfat analysis is not generally affected by surfactants, because such materials are not likely to contribute C_4, C_6, and C_8 acids when hydrolyzed. The major exceptions are C_6 and C_8 acids from coconut and palm kernel sources. However, their presence could be inferred by higher than expected Polenske values (2) based on the Reichert value.

If the analysis required separate quantitation for butterfat and cocoa fat or the surfactants, the fats are easily separated from the surfactants via column chromatography, as noted in Table 4. For Table 4, identification of butter and cocoa fat fractions was based on IR (11) comparison with authentic butterfat. RMPK numbers were obtained to determine per cent butterfat. The lecithin fraction was identified by IR comparison with commercial lecithin; positive N, positive P (12); and surfactant dye transfer test (13). The Soxhlet-extracted fractions were chiefly long chain fatty acids and their sodium salts, inferred from IR and the sodium flame test. These fractions were treated with warm 20% tetramethylammonium hydroxide in methanol (14). The fatty acid methyl esters formed thereby and in the injector port were identified in routine fashion by GLC-MS (15). A typical analysis of the fatty acids (principal peaks, area per cent) is: C_{12}, 2.6%; C_{14}, 8.7%; C_{16}, 31.3%; $C_{18:0}$, 22.0%; $C_{18:1}$, 35.4%. Some commercial coconut oil-based surfactants were used for concurrent IR and GLC analyses, thereby excluding coconut oil as the basic oil source. Reference to GLC data for commercial basic oils (16) did not allow assignment to a single oil source for this surfactant fraction.

Table 5 shows the distribution of butterfat and cocoa fat for the samples which were separated by column chromatography. The percentages are based on the total of lipid material and surfactants, but the RMPK values give only the butterfat, which should therefore be less than the total fats isolated by chromatography. The remaining percentage of fatty material is ascribed to cocoa butter, a stated ingredient.

The proposed method, extraction with refluxing $CHCl_3$–methanol (70+30) for 15 hr, is expected to render tractable those dried or friable food products which contain sufficient surfactants to make the current AOAC methods inefficient or ineffective. It is suggested that some final treatment, e.g., **16.232(b)** (5), be applied to the extracted residue to check that all the lipid material is extracted by the $CHCl_3$–methanol.

Table 5. Results of chromatographic separation of lipidic material

Sample	Total fats, %[a]	Butter-fat, %[b]	Cocoa fat, %[c]	Material recovered %[d]
Cocolac	86.12	80.37	5.75	97.75
Cocolac	85.20	80.37	4.89	99.12
Milo (Australia)	92.66	86.69	5.97	98.48
Milo (Jamaica)	62.63	57.09	5.54	80.04[e]

[a] Based on weight of fat fractions separated by alumina column chromatography.

[b] From Reichert-Meissl, Polenske, and Kirschner values.

[c] By difference of preceding values.

[d] Based on material applied to column.

[e] The weight loss is believed to be surfactants retained on the alumina, which was not Soxhlet-extracted.

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INDUSTRIAL CHEMICALS

Gas-Liquid Chromatographic Determination of Chlorinated Benzenes and Phenols in Selected Biological Matrices

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The determination of higher chlorinated benzenes and chlorinated phenols at parts per billion or parts per million levels in biological samples is described. The method includes sulfuric acid digestion, silica gel column chromatography, methylation, alumina column chromatography, and detection by electron capture gas chromatography. Recoveries are reported for fish flesh, rabbit tissues, and duck tissues with detection limits of 10–15 ppb (ng/g tissue).

Millions of pounds of chlorobenzenes and chlorophenols are produced annually by chemical companies in the United States. Chlorinated benzenes are used as process solvents, starting materials, or intermediates for the manufacture of other chemical products such as pesticides, phenols, and dyestuffs. Chlorinated phenols are widely used as fungicides, antimicrobials, wood preservatives, and intermediates in the production of many agricultural products. The uses, determination, toxicity, and environmental fate of pentachlorophenol (1) and chlorobenzenes (2–7) have been described. Misuse of these compounds and improper disposal practices have resulted in contamination of watersheds (8, 9) and the food chain (10, 11).

The concentration and persistance of chlorinated benzene or chlorinated phenol residues in biological samples are of interest. Most environmental studies, toxicity studies, and analytical methodology have dealt with hexachlorobenzene, pentachlorophenol, and related pesticides. Highly chlorinated benzenes and phenols have been determined by using electron capture gas-liquid chromatography (GLC) because of its high sensitivity. Most methods for chlorobenzenes rely on an electron capture GLC screening procedure for organochlorines, with confirmation on GLC columns of different polarity (11–13). Some methods specify a Florisil and/or charcoal sample cleanup for biological samples (10, 14, 15). Chlorophenols have been determined by GLC with electron capture detection following derivatization with trimethylchlorosilane (16), acetic anhydride (8, 17, 18), diazomethane (19), or diazoethane (20, 21). Negative ion chemical ionization mass spectrometry has also been used for this determination (22). Cleanup by silica gel and gel permeation chromatography has been used.

A method is described here for determining higher chlorinated benzenes (tri-, tetra-, penta-, and hexachlorobenzenes) and higher chlorinated phenols (tri-, tetra-, and pentachlorophenols) in fish flesh, rabbit tissues, and duck tissues. We extended a method for determining pentachlorophenol in bovine milk (23) to include chlorobenzenes and other chlorophenols. Before application of this methodology to environmental samples, recovery studies were carried out to define its accuracy, precision, and minimum detection limits.

METHOD

Reagents and Apparatus

(a) *Solvents.* — Distilled-in-Glass (Burdick & Jackson Laboratories, Muskegon, MI 49442).

(b) *Sulfuric acid–water (1+1).*—Add reagent grade H_2SO_4 (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) to an equal volume of deionized water.

(c) *Silica gel.*—100–200 mesh Bio-Sil A (Bio-Rad Laboratories, Richmond, CA 94804). Dry at 120°C for 6 hr; then deactivate with 5% (w/w) water.

(d) *Acid alumina.*—100–200 mesh Bio-Rad acid alumina AG4. Use as received.

(e) *Diazomethane.*—Prepare from Diazald® reagent and diazomethane generation kit (Z10, 025–0) obtained from Aldrich Chemical Co., Milwaukee, WI 53233.

(f) *Liquid chromatographic columns.*—Pack 10 cm × 15 mm glass disposable transfer pipets with adsorbents for liquid chromatographic cleanup steps.

(g) *Kuderna - Danish concentrator.* — (Kontes Glass Co., Vineland, NJ 98360.) Equipped with 10 mL concentrator tube (K-570050) and small scale

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Table 1. Recovery[a] of chlorobenzenes and chlorophenols added to catfish flesh

Compound	Added, ng/g	Rec., %	Std dev.
1,2,4-Cl_3-Benzene	19–78	76	15
1,2,3-Cl_3-Benzene	12–48	75	13
1,2,4,5-Cl_4-Benzene	15–60	79	8
1,2,3,4-Cl_4-Benzene	26–104	82	7
Cl_5-Benzene	9–37	89	6
Cl_6-Benzene	8–34	93	6
2,4,6-Cl_3-Phenol	19–75	90	12
2,4,5-Cl_3-Phenol	23–93	90	7
2,4,6-Br_3-Phenol	12–50	86	8
2,3,4,6-Cl_4-Phenol	14–54	88	7
2,3,4,5-Cl_4-Phenol	21–84	92	7
Cl_5-Phenol	9–37	99	11

[a] Each recovery is the mean of 9 samples.

3-stage Snyder column (K-569001).

(h) *Gas chromatograph.* — Hewlett - Packard Model 5713A equipped with ^{63}Ni linear electron capture detector. Operating conditions: temperatures (°C)—injector 200, column programmed from 100 to 200 at 8°/min, detector 300; nitrogen carrier gas flow 34 mL/min.

(i) *Column.*—300 cm × 2 mm glass packed with 0.5% Silar 10C coated on specially deactivated support of 80–100 mesh Chromosorb W(AW).

Preparation of Sample

Weigh 5 g tissue into clean 2 oz wide-mouth bottle. Add 10 mL benzene-hexane (20+80) and 25 mL H_2SO_4–water (1+1). Cap tightly and let digest overnight at room temperature. After digestion, transfer sample to 60 mL separatory funnel and shake gently 2 min. Transfer organic layer to 25 mL mixing cylinder, and re-extract acid phase with three 5 mL portions of benzene–hexane (20+80). Combine all extracts in mixing cylinder and adjust volume to 25 mL.

Pack disposable pipet column with small plug of glass wool and 5 cm bed of Bio-Sil A silica gel (5% w/w water) and prewash with 10 mL benzene–hexane (20+80). Elute 10 mL sample extract (equivalent to 2 g tissue) through silica gel column and collect eluate; then elute column with 10 mL benzene and combine both fractions. Add 1 mL methanol to eluates, and methylate with diazomethane. Remove excess diazomethane under stream of nitrogen and concentrate solvent to 1 mL in Kuderna-Danish concentrator. Add 4 mL hexane to concentrator tube, mix, and place solution on column of disposable pipet packed with 5 cm bed of acid alumina AG4. Collect eluate in 10 mL Kuderna-Danish concentrator tube and elute column with additional 5 mL benzene–hexane (20+80). Combine both fractions and concentrate to 1 mL as before. Analyze extract by GLC with electron capture detection. Compare sample responses to those of standards of known concentration for quantitation purposes.

Results and Discussion

Tissue fortification experiments were initially conducted at levels of 10–100 ppb because these were expected concentration ranges for environmental samples. Tissues were spiked by adding aliquots of stock solutions in hexane of the chlorinated benzenes and phenols to the tissues at parts per billion levels before addition of the sulfuric acid. These recovery data are shown in Tables 1–3. Later, to test the applicability of this method to analysis of highly contaminated samples such as would be encountered in a feeding study, tissues were fortified at much higher concentration levels (0.5–1000 ppm). As shown from the recovery data (Table 4), the sample preparation procedure is equally applicable to parts per billion and parts per million levels of these compounds.

An important feature of this analysis is the derivatization of the chlorophenols to chloroanisoles before the final liquid chromatographic step. This allows the use of a much less polar solvent to elute the compounds from the acid alumina column. The chromatograms in Figs 1–3 show that the final residue is cleaner when methylation precedes the final chromatographic purification step. Although the chromatogram in Fig. 3 has some small interference peaks, the

Table 2. Recovery of chlorobenzenes and pentachlorophenol added to rabbit tissues

	Fat				Liver			
Compound	Added, ng/g	No. of detns	Rec., %	Std dev.	Added, ng/g	No. of detns	Rec., %	Std dev.
1,2,4,5-Cl_4-Benzene	10–100	6	95	12	10–100	4	83	7
1,2,3,4-Cl_4-Benzene	10–100	7	94	8	10–100	4	82	9
Cl_5-Benzene	10–100	8	92	6	10–100	4	89	4
Cl_6-Benzene	10–100	8	89	7	10–100	4	94	8
Cl_5-Phenol	10–100	8	90	7	10–100	4	83	5

Table 3. Recovery[a] of chlorobenzenes and pentachlorophenol added to duck tissues

Compound	Muscle			Brain			Fat			Serum			Liver		
	Added, ng/g	Rec., %	Std dev.	Added, ng/g	Rec., %	Std dev.	Added, ng/g	Rec., %	Std dev.	Added, ng/g	Rec., %	Std dev.	Added, ng/g	Rec., %	Std dev.
1,2,4-Cl_3-Benzene	100	81	13	100	78	11	70	87	3	40	79	9	100	68	3
1,2,4,5-Cl_4-Benzene	100	77	5	100	80	3	140	87	4	40	82	5	100	77	3
1,2,3,4-Cl_4-Benzene	100	87	14	100	90	7	440	86	3	40	93	9	100	84	3
Cl_5-Benzene	30	93	10	30	95	6	160	88	3	20	87	7	30	88	11
Cl_6-Benzene	30	86	5	30	90	4	20	66	7	20	65	5	30	88	4
Cl_5-Phenol	100	93	10	100	92	5	20	113	17	50	104	10	100	95	8

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

Table 4. Recovery[a] of chlorobenzenes and pentachlorophenol added to duck tissues

Compound	Fat			Serum			Liver			Muscle			Brain		
	Added, μg/g	Rec., %	Std dev.	Added, μg/g	Rec., %	Std dev.	Added, μg/g	Rec., %	Std dev.	Added, μg/g	Rec., %	Std dev.	Added, μg/g	Rec., %	Std dev.
1,2,4-Cl_3-Benzene	150	110	5	0.47	96	4	3.0	97	5	3.0	97	4	2	114	6
1,2,4,5-Cl_4-Benzene	310	95	4	1.0	94	4	8.3	83	3	6.2	84	4	5.2	86	4
1,2,3,4-Cl_4-Benzene	1000	94	4	4.9	92	4	22	90	5	18	94	2	12	94	5
Cl_5-Benzene	300	92	3	0.82	90	5	8	84	3	7	85	4	5	84	6
Cl_6-Benzene	42	94	5	0.12	88	6	1.1	82	7	1.1	82	7	1.1	83	11
Cl_5-Phenol	1.8	76	3	6.8	91	6	1.2	95	5	1.2	95	5	1.2	106	11

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

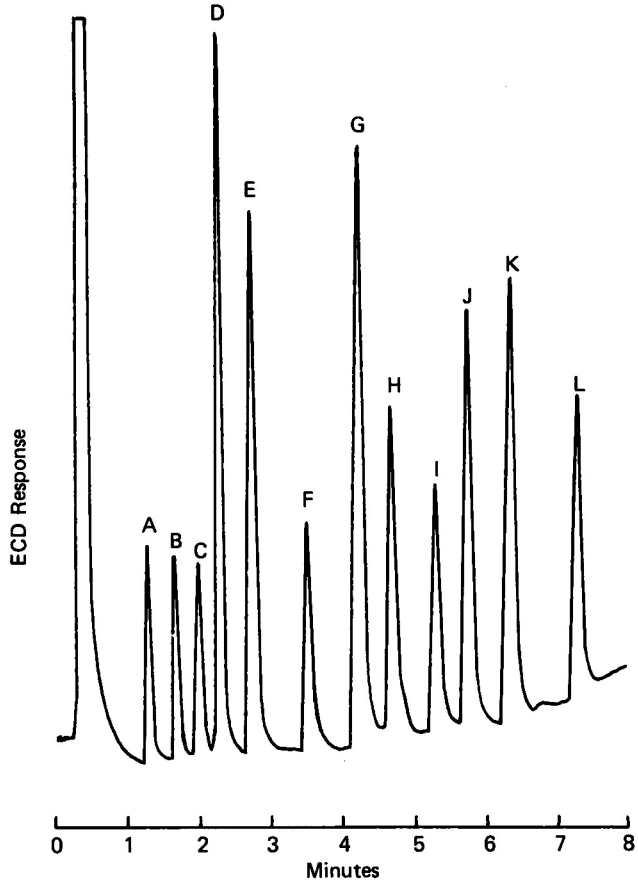

FIG. 1—Gas chromatogram of calibration standard.

Peak identification: A, 1,2,4-trichlorobenzene 6.0 ppb; B, 1,2,3-trichlorobenzene 9.7 ppb; C, 1,2,4,5-tetrachlorobenzene 7.5 ppb; D, 2,4,6-trichloroanisole 9.4 ppb; E, 1,2,3,4-tetrachlorobenzene 13.0 ppb; F, pentachlorobenzene 4.6 ppb; G, 2,3,4,6-tetrachloroanisole 6.8 ppb; H, 2,4,5-trichloroanisole 12.0 ppb; I, hexachlorobenzene 4.2 ppb; J, 2,4,6-tribromoanisole 4.6 ppb; K, pentachloroanisole 4.6 ppb; L, 2,3,4,5-tetrachloroanisole 11.0 ppb.

low concentrations of the compounds observed (1–10 ppb) should be noted.

The presence of the more volatile chlorobenzenes and chlorophenols was often observed in reagent blanks that were analyzed concurrently with fortified samples. The volatility of these compounds makes them very difficult to exclude from reagents when low concentrations may be present in the laboratory. To minimize the reagent blank contamination, adsorbents were Soxhlet-extracted with methylene chloride for 4 hr before activation. Reagent blanks were always analyzed with samples and results were corrected for any reagent blank responses.

In the procedure described, there is no differentiation between the chlorophenols and corresponding chloroanisole content. If individual concentrations are required, as in metabolism studies, inclusion of an additional acid alumina column before methylation, and elution of the chlorobenzenes and chloroanisoles with benzene–hexane (20+80) and the underivatized chlorophenols with benzene, will provide these data.

The analytical procedure described will provide reliable data for determining chlorobenzenes and chlorophenols in a variety of biological matrices without the use of sophisticated instrumentation such as gas chromatography–mass spectrometry.

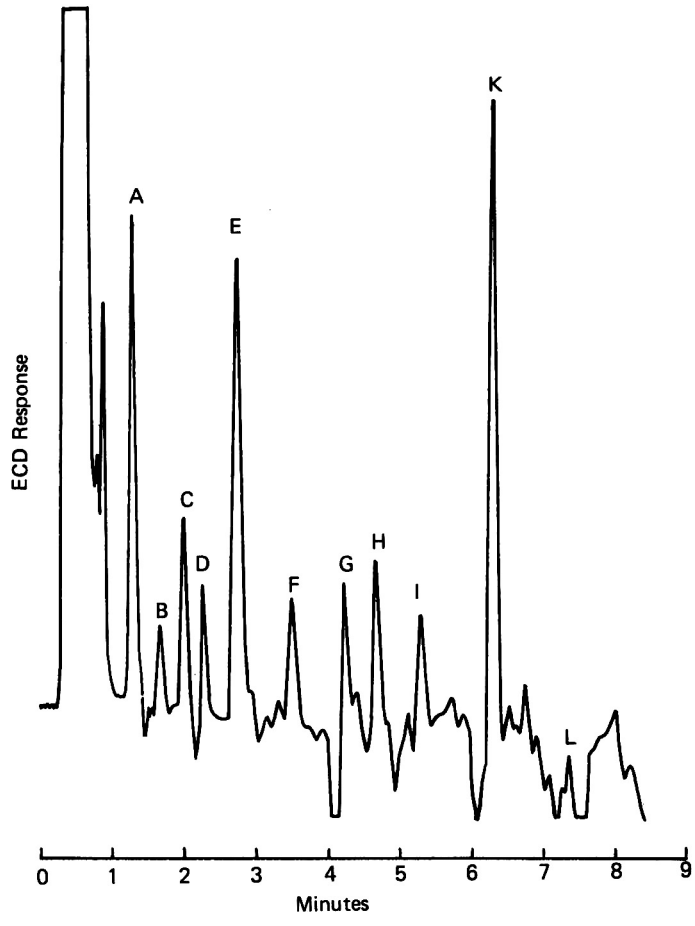

FIG. 2—Gas chromatogram of fish residue chromatographed on acid alumina before methylation. See Fig. 1 for peak identification.

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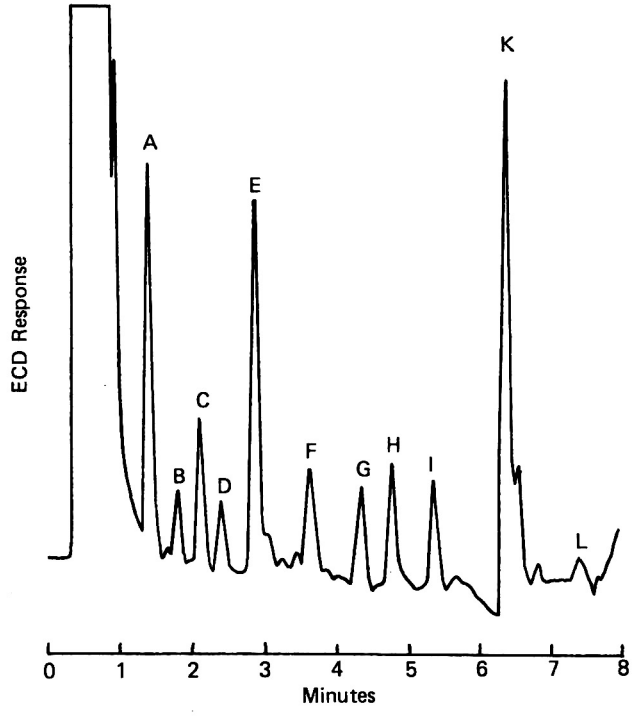

FIG. 3—Gas chromatogram of fish residue prepared according to the described procedure. See Fig. 1 for peak identification.

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PESTICIDES

Gas-Liquid Chromatographic Determination of Dioxathion and Quintiofos in Organophosphorus Dip Washes

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A rapid method for the analysis of dip washes is described which eliminates the usual solvent extraction procedure. The dip wash is initially diluted with acetone and then with petroleum ether. The diluted dip wash is analyzed by gas-liquid chromatography, using an alkali ionization detector sensitive to phosphorus compounds. The method was applied to the determination of dioxathion (2,3-*p*-dioxanedithiol *S,S*-bis(*O,O*-diethyl phosphorodithioate)), and quintiofos (*O*-ethyl *O*-8-quinolyl phenylphosphonothioate) dip washes. Average recoveries from fouled dip washes were 100 and 104%. GLC of these compounds with an internal standard is described, which improves the precision of the method to ±2%.

The increasing number of organophosphorus dip samples received for routine analysis at the Veterinary Research Laboratory, Kabete, Kenya, has called for simplification of the preparative procedures which are necessary before determination by gas-liquid chromatography (GLC).

Most organophosphorus acaricides are insoluble in water and are therefore formulated as oil emulsions. The conventional method for dip wash analysis is to extract the acaricide with water-immiscible solvents. This process usually requires shaking the sample with solvent for some time (some methods require 1–2 hr) to remove adsorbed acaricide from mud, hair, and other debris present in the sample, followed by centrifugation. Extraction may not be complete even with clean dip samples, because the properties of the emulsifying agents used in the acaricide formulations may be difficult to degrade. Various methods for determining the organophosphorus compounds in the solvent extract include thin layer chromatography (1), total phosphorus estimation (2, 3), and GLC. The latter method is the most sensitive, especially when an alkali ionization detector is used (4). Organophosphorus compounds in solution can be detected at concentrations of 1×10^{9} g/μL or 1 ng/μL and less. For dip wash samples containing the recommended levels of 0.02–0.05% (w/v), the solvent extract from an equal volume of dip sample can be diluted 50–100 times for analysis.

A method is described here in which the dip wash samples are simply diluted with a water-miscible solvent before analysis by GLC. The method has been applied with particular reference to dioxathion (2,3-*p*-dioxanedithiol *S,S*-bis(*O,O*-diethyl phosphorodithioate), Delnav®), and quintiofos (*O*-ethyl *O*-8-quinolyl phenylphosphonothioate, Bacdip®)—the organophosphorus acaricides which are currently recommended for tick control in Kenya. Once the GLC conditions are established, the method may be applied to any other organophosphorus dip wash.

METHOD

Apparatus

(a) *Gas chromatograph.* — Pye Series 104 equipped with alkali flame detector with rubidium chloride annulus, and S4 autojector, or equivalent (Pye Unicam Ltd, Cambridge, UK).

Chromatography conditions: (*1*) Dioxathion: glass column, 1 m × 4 mm id, packed with 5% OV-17 on 80–100 mesh Gas-Chrom Q; column 235°C, injection port 250°C, detector 240°C. (*2*) Quintiofos: glass column, 0.3 m × 4 mm id, packed with 2% OV-17 on 80–100 mesh Gas-Chrom Q; column 235°C, injection port 235°C, detector 240°C. Flow rates for either column: nitrogen carrier 50 mL/min, hydrogen 30 mL/min, air 500–600 mL/min.

(b) *Potentiometric recorder.*—1 mV Honeywell 194, or equivalent (Honeywell Ltd, Brentford, Middlesex, UK).

(c) *Integrator.* — Hewlett-Packard HP 3380A (Hewlett-Packard, Avondale, PA 19311) (optional).

(d) *Dispensers for organic solvents.*—Repipet® No. 3050-A-L (Labindustries, 620 Hearst Ave, Berkeley, CA 94710).

Reagents

(a) *Petroleum ether.*—60–80°C bp.

(b) *Quintiofos.*—Technical grade of known concentration used in preparation of formulation of Bacdip.

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(c) *Technical carbophenothion.*—Internal standard for quintiofos, 1 mg/mL petroleum ether. Working solution: Dilute 2.5 mL to 1 L with petroleum ether (adjust as necessary).

(d) *Technical Delnav.*—Specification as used in formulation.

(e) *Technical fenitrothion.*—Internal standard for dioxathion, 1 mg/mL acetone. Working solution: Dilute 2.5 mL to 1 L with petroleum ether (adjust as necessary).

Preparation of Dip Samples and Standards

Shake dip sample to ensure even distribution of dirt. Pipet 10 mL sample into 50 mL cylinder or flask and dilute to volume with acetone, using dispenser. Stopper and shake by hand for 2 min. Let settle until clear. Prepare standard solutions of acaricides in acetone at known concentrations (% w/v) approximating recommended strengths of dip wash. Pipet 10 mL into 50 mL cylinders or flasks, add 10 mL distilled water, and dilute to volume with acetone. Stopper and shake.

Pipet 5 mL acetone dilutions into 50 mL flasks or cylinders (or 1 mL to 10 mL) and dilute to volume, using dispenser with petroleum ether containing appropriate internal standard. Shake and let small amount of water present separate. Analyze clear petroleum ether solutions by GLC. Inject standard at frequent intervals between samples to check performance of system.

Before use, gas-liquid chromatograph should be checked for linearity by chromatography of standards over range of concentrations. Concentration of internal standard working solution should be adjusted so that peak height (PH) response is similar to external standard used for assessment.

Calculation

The concentrations of Delnav and quintiofos in dip samples are calculated by determining the response of the particular compound in comparison with the response of the standard. Response values for standard and samples are corrected for variation in injection volume by the use of an internal standard. The internal standard is given an arbitrary value such as 1 or 10, and the corrected values are calculated:

$$\text{Concn} = (A/B) \times 1$$

where A = peak height or area of Delnav (as dioxene) or quintiofos, and B = peak height or area of internal standard (fenitrothion or carbophenothion).

A factor (F) is calculated:

$$F = \frac{\%\ (w/v)\ \text{concentration of standard (Delnav or quintiofos)}}{\text{corrected value of standard}}$$

The corrected values of the samples are then multiplied by F to obtain concentration % (w/v) of Delnav or quintiofos in the dip wash.

Results

Recovery of an acaricide from clean dip samples is complete using the acetone dilution method because the acetone is completely miscible with the dip sample. For spiked dip samples containing a high proportion of dirt, the acetone dilution method gave average recoveries of 100% Delnav and 104% quintiofos (Table 1). Table 2 shows the comparative analyses of 6 Delnav and Bacdip dip wash samples by using the above method and a conventional benzene extraction method. In most cases the acetone dilution method gave slightly higher results; in some cases considerably higher results were obtained.

Even with an automatic sampling device, an internal standard was necessary to correct for variation in injection volume. This can be seen by the values for peak height given in Table 3,

Table 1. Recovery of dioxathion and quintiofos from spiked dip samples

Dip	Concn	Total	Found	Rec., %
	Dioxathion,[a] ppm			
1	321	443	427	96
2	221	352	341	97
3	239	369	366	99
4	92	235	244	103
5	425	538	532	99
6	501	607	585	96
7	666	757	794	105
8	428	540	531	98
9	494	600	614	102
10	404	519	533	103
Av.				100
	Quintiofos,[b] ppm			
11	187	217[b]	228	105
12	158	191	202	106
13	159	192	189	99
14	88	127	118	93
15	26	71	82	115
16	197	226	218	96
17	192	222	238	108
18	55	97	104	107
19	44	87	92	106
20	252	276	293	106
Av.				104

[a] 50 mL dip wash spiked with 5 mL of an aqueous suspension of Delnav emulsifiable concentrate containing 0.167% dioxathion.

[b] 50 mL dip wash spiked with 5 mL of an aqueous suspension of Bacdip emulsifiable concentrate containing 0.0517% quintiofos.

Table 2. Comparative analyses of dip samples, using acetone dilution method and benzene extraction method

Dip	Acetone diln	Benzene extn
	Delnav, ppm	
21	668	684
22	828	812
23	261	245
24	452	488
25	443	393
26	500	481
	Bacdip, ppm	
27	82	77
28	200	200
29	393	376
30	187	181
31	303	313
32	277	277

Table 3. Peak height response (cm) of analyte and appropriate internal standard

Int. std	Analyte	Corrected response values[a]
	Dioxene (fenitrothion int. std)	
9.5	10.2	10.74
9.5	9.2	10.56
8.9	9.4	10.56
7.95	8.45	10.63
7.4	7.9	10.68
7.55	8.05	10.66
7.4	7.8	10.54
7.1	7.4	10.42
	Quintiofos (carbophenothion int. std)	
7.15	7.5	10.49
9.40	7.85	10.48
11.25	11.80	10.49
10.90	11.40	10.46
11.00	11.50	10.45
10.10	10.50	10.40
11.15	11.70	10.49
10.25	10.85	10.57

[a] Internal standard = 10.

which are typical results for the same standard solution injected between every 5 samples. With the internal standard, corrected values of the external standard are mostly within ± 2% of the mean. Samples need only be injected once. However, gradual deposition of alkali salt on the probe of an alkali ionization detector may occur, and this affects the relative response of different organophosphorus compounds. When corrected values for the external standard increase or decrease significantly, the probe is cleaned.

Discussion

The acetone solutions for GLC are diluted with petroleum ether, because acetone appeared to have a corroding effect on the syringe plunger, which eventually affected the free movement within the barrel. Dilution can be made with acetone if this does not affect the performance of the syringe.

The petroleum ether used for dilution can be recovered. The solvent is allowed to settle in a separating funnel and the water layer is drained. Traces of water are removed by adding anhydrous sodium sulfate. Dried solvent is then filtered into a distillation flask and distilled; the first and last fractions are rejected. The recovered solvent is finally checked by GLC to ensure that no residues of organophosphorus compounds are present.

GLC of Delnav poses special problems. The technical material consists of 68–75% dioxathion as a mixture of *cis*- and *trans*-isomers: about 24% *cis*-, 48% *trans*-, and 30% related compounds (Hercules Inc.). Analysis of Delnav (dioxathion) in dip washes by GLC was first described by Paterson (5). However, the peak attributed to the *trans*-isomer and used as a measure of the dioxathion concentration was in fact a breakdown product, dioxene (2-*p*-dioxenethiol-*S*-*O*,*O*-diethyl phosphorodithioate), which occurred in the heated inlet of the gas chromatograph (private communications, Wellcome Research Laboratories, Berkamstead, Herts., UK, 1970 and 1977; Hercules Inc., Wilmington, DE 19899, 1978). A high inlet (at least 240°) and relatively high column temperature is important for maximum GLC response. There are some reservations about the GLC method for the quantitative analysis of Delnav. However, it has been shown in this laboratory, by the recovery experiments and by the comparison of the GLC method with the total phosphorus estimations that GLC is suitable for monitoring Delnav dip washes. The gas-liquid chromatogram of dioxathion (as dioxene) and fenitrothion is shown in Fig. 1.

The GLC of quintiofos was also investigated. A number of different columns and operating conditions were tried. It was convenient to reduce the analysis time to <5 min by using a 0.3 m column packed with 2% OV-17 on Gas-Chrom Q at 235°C (Fig. 2).

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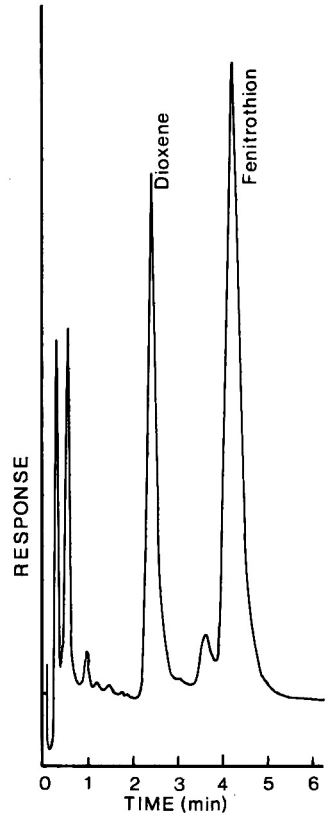

FIG. 1—Gas-liquid chromatogram of dioxathion, as dioxene, and internal standard fenitrothion. See text for GLC column and conditions.

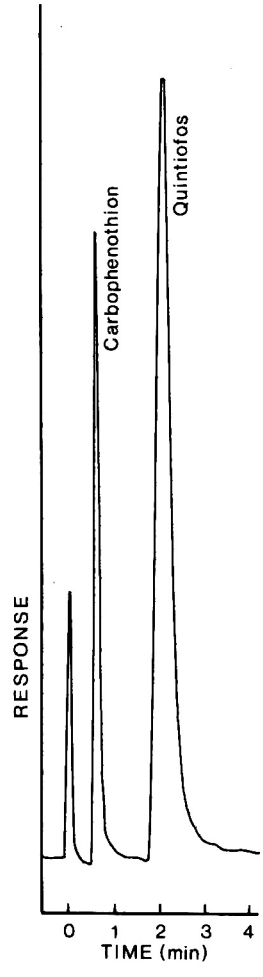

FIG. 2—Gas-liquid chromatogram of quintiofos and internal standard carbophenothion. See text for GLC column and conditions.

Acknowledgment

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Gas-Liquid Chromatographic Determination of Mirex and Photomirex in the Presence of Polychlorinated Biphenyls: Interlaboratory Study

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Mirex and photomirex (8-monohydromirex) were separated from polychlorinated biphenyls (PCBs) and other aromatic compounds by nitration with fuming nitric acid–concentrated sulfuric acid and removal of nitro-PCBs on an alumina microcolumn; the compounds were then determined by gas-liquid chromatography. Recoveries of Mirex and photomirex were 102±8 and 104±5%, respectively, from standard solutions which had a PCB-to-Mirex and photomirex ratio of 1000. Recoveries from fortified, uncontaminated samples of sediment, fish, and eggs averaged 93±7 and 92±3% for Mirex and photomirex, respectively. The coefficients of variation for repeatability and reproducibility averaged 8 and 15%, respectively, in an interlaboratory study conducted by 4 laboratories using extracts of naturally contaminated substrates (sediment, carp, eel, and gull egg). Levels of Mirex in the samples ranged from 0.1 to 8 mg/kg, and levels of PCB ranged from 0.5 to 166 mg/kg.

Mirex and photomirex (8-monohydromirex) have been identified as major contaminants in Lake Ontario biota (1). Photomirex, in particular, is difficult to determine in the presence of large quantities of polychlorinated biphenyls (PCBs) without prior separation because the retention time is similar to that of major heptachlorobiphenyls on most gas-liquid chromatographic (GLC) columns. Initial efforts to develop a method which could be used for the routine determination of both Mirex and photomirex centered around the selective response to chloroalkanes of the Hall electrolytic conductivity detector (1). A suitable choice of operating conditions allowed the determination of Mirex and photomirex in the presence of 50-fold or less excess of PCBs. At higher PCB/Mirex ratios, this method was unreliable. The sensitivity was also poor because the practical detection limit for Mirex and photomirex was about 1×10^{-9} g under the conditions used. Charcoal column chromatography has been used (2) to separate Mirex from PCBs, but photomirex eluted largely in the PCB fraction. The polyurethane foam/charcoal column method of Stalling *et al.* (3) separates Mirex and photomirex from PCBs in spiked sediments (4). This method may be of general use, but was not tested at high PCB/Mirex ratios or on naturally contaminated samples.

Lewis *et al.* (5) showed that photolysis in the presence of diethylamine selectively eliminates PCB interference in PBC/Mirex mixtures. However, some interferences appear to remain in the photomirex and earlier regions of the chromatogram. In addition, potentially interfering non-PCB aromatic compounds such as *p,p'*-DDT are not destroyed by the method. Chemical alteration of chloroaromatic compounds by nitration with concentrated H_2SO_4–fuming HNO_3 (1+1) (6–8), which is used as a method for the confirmation of Mirex in the presence of other organochlorine pesticides and PCBs (9), appeared to offer a better route to a rapid and sensitive method for Mirex and photomirex determination. Of the commonly occurring organochlorine residues, only chlordane and nonachlor isomers, HCB, Mirex, and photomirex were recovered after nitration (9). A scaled-down nitration and cleanup method was accordingly developed. This method is described here along with the results of an interlaboratory study among the authors' laboratories using extracts of carp, eel, sediment, and herring gull egg from Lake Ontario.

METHOD

Apparatus and Reagents

(a) *Centrifuge tubes.*—Glass, conical, 15 mL, with glass stoppers.

(b) *Disposable microcolumn.*—Pasteur pipet, short tip, 6 mm od, minimum barrel length 80 mm. Plug end with hexane-rinsed glass wool.

(c) *Gas-liquid chromatograph.*—Hewlett-Packard 5730 with linear ^{63}Ni electron capture detec-

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tor, or equivalent; 180 cm × 2 mm id glass column packed with 1% SP-2100 + 2% SP-2401 on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA), or equivalent; oven temperature 200°C, injection port temperature 250°C, detector temperature 300°C; methane–argon (10+80) carrier gas at 40 mL/min. Other stationary phases may be used (i.e., SP-2100), because Mirex and photomirex are adequately separated with all phases tested. However, if a non-polar phase (SP-2100, OV-1, OV-101) is used, absence of interference, particularly with photomirex, should be checked by analysis of a nitrated extract on a more polar phase.

(d) *Sodium sulfate.*—Anhydrous, granular. Heat or solvent-treat to remove interferences.

(e) *Solvents.* — Dichloromethane–hexane (20+80), pesticide analysis reagent grade (Caledon Laboratories Inc., Toronto, Ontario, Canada).

(f) *Photomirex.*—Prepared from Mirex as given by Hallett *et al.* (10).

(g) *Alumina.*—Fisher neutral, Brockmann activity 1, 80–200 mesh, or equivalent. Activate ≥2 hr at 550°C, deactivate 1% by weight with water, let equilibrate 24 hr. Constancy of activity depends on tightness of storage container, length of exposure to air on handling, and humidity in air. With reasonable precaution, activity should remain constant for several weeks.

Extraction and Cleanup

Extract sample by method which isolates total lipid. Acetonitrile partitioning procedure (**29.014**, 11) or any other method which involves partitioning between hexane or petroleum ether and acetonitrile–water, such as that of Porter and Burke (12) should not be used without modification. Because of unfavorable partition coefficients, Mirex recovery can be seriously reduced unless water is excluded (13), or a small percentage of dichloromethane is added to the hexane (14). Gel permeation chromatography cleanup (15) is suitable for removing most lipid.

Complete cleanup on Florisil column, such as given in sec. **29.015** (11), *Pesticide Analytical Manual* (16), or *Analytical Methods for Pesticide Residues in Foods* (19). Adjust volume of first eluting solvent (hexane or petroleum ether) to collect only PCBs, DDE, HCB, and Mirex, but not *p,p'*-DDT (17). This eliminates any potential coelution of lipids. Lipids may cause low recoveries of Mirex when present during nitration.

Sediment extracts should be treated to remove sulfur before nitration, for example, by shaking with copper powder which has been rinsed with dilute HCl followed by distilled water, acetone, and hexane.

Nitration and Alumina Chromatography

Pipet 1–10 mL aliquot of hexane fraction from Florisil column into 15 mL centrifuge tube. Up to 200 μg PCB may be nitrated. Add 1 drop 10% mineral oil in hexane and evaporate almost to dryness under clean air or nitrogen stream. Rinse tube with 1 mL hexane and evaporate solvent to dryness. Add 0.5 mL fuming HNO_3 followed by 0.5 mL concentrated H_2SO_4. Mix thoroughly using vortex mixer, and insert glass stopper. Place stoppered tube in 70°C water bath for 30 min. Cool tubes in freezer or ice bath; add 2 mL ice water (pre-extracted with hexane). *Caution:* Be extremely careful to guard against violent ejection of acid from tube during this step (wear protective goggles and rubber gloves). Let come to room temperature. Correct performance of addition of water is critical to good recoveries of Mirex and photomirex. If water-acid layers are not quickly mixed, localized heating may cause Mirex to be steam-distilled from tube. Water must be added either quickly by pipet with continuous vortex mixing, or very slowly with vortex mixing and cooling before each addition.

Pipet 4 mL dichloromethane–hexane (20+80) into tube; mix with vortex mixer for 30 sec with stopper removed. After separation of layers, pipet 2 mL organic layer into another centrifuge tube, and evaporate just to dryness. Redissolve residue in 1 mL hexane. Prepare alumina column by adding 2.5 cm deactivated alumina and 3–5 mm Na_2SO_4 to Pasteur pipet column. If humidity is high, column must be used immediately. Premeasure 15 mL hexane and transfer nitrated residue to alumina column with Pasteur pipet, rinsing tube with small portions of hexane. Continue adding hexane to column until 15 mL has been transferred, not allowing meniscus to drop below Na_2SO_4 layer. Use suitably sized volumetric glassware as receiver, dilute to volume, and analyze by GLC.

Interlaboratory Study

Each of the 4 laboratories prepared an extract of one substrate known to contain Mirex and photomirex and determined recoveries in the nitration procedure using similar substrates fortified with Mirex and photomirex. The extract was then sent, in a sealed ampoule, to each of the other laboratories. The photomirex and Mirex standard solutions were distributed in sealed ampoules from one laboratory. Each laboratory analyzed each substrate 4 times using its own cleanup procedures, but following the prescribed nitration and alumina chromatography procedure. Laboratory 1 used a 1.2% deactivated Florisil column for cleanup (17); Laboratory 2 used a modified Langlois *et al.* (18) cleanup procedure (5% deactivated Florisil and 250 mL dichloromethane-hexane (20+80)), followed by elution with 60 mL hexane on a 1 cm × 30 cm column of 1.5% deactivated

Florisil; Laboratory 3 used 2.5% deactivated Florisil and 300 mL hexane eluate (19); Laboratory 4 used a modified Langlois *et al.* (18) procedure (5% deactivated Florisil and 300 mL dichloromethane-hexane (20+80)).

Results and Discussion

Preliminary results showed that a single extraction of the HNO_3–H_2SO_4–water layer with hexane after nitration was insufficient for complete recovery of Mirex (83%). Photomirex was more efficiently partitioned (90%). Addition of a small percentage of dichloromethane to the hexane (10%) improved the recovery of Mirex to 93±4%. A level of 20% dichloromethane in hexane was chosen for the procedure because this mixture gave good recoveries (100 ± 1%), remained as the upper phase, and did not evaporate significantly during vortex mixing. The dichloromethane must be completely removed before the alumina chromatography, or nitro-PCBs may be eluted.

The elution profile of Mirex and photomirex from the alumina column is shown in Fig. 1. In spite of the small size of the column, Mirex and photomirex are partially resolved from each other. An elution volume of 15 mL is usually sufficient for 99% recovery of both compounds. Nitrated PCBs (as evidenced by broad, late-eluting GC peaks) do not appear in the eluate until approximately 25 mL has been eluted. Elution with 15 mL hexane is therefore adequate to ensure complete recovery of Mirex and photomirex without interfering nitro-PCBs, even if the activity of the alumina should decrease slightly during preparation and use of the column. Under highly humid conditions, it may be necessary to use activated alumina and less hexane.

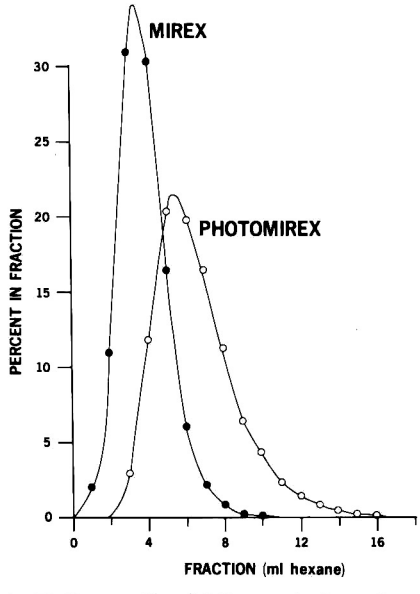

FIG. 1—Elution profile of Mirex and photomirex from alumina microcolumn (0.5 g, 1% deactivated alumina in Pasteur pipet).

Table 1. Recovery of Mirex and photomirex (mean±SD of 3 replicates) after nitration of fortified PCB (1:1 Aroclor 1254:1260) standard solutions

Amount nitrated, μg				Rec., %	
PCB	Mirex	Photo-mirex	PCB/ Mirex	Mirex	Photo-mirex
0	0.2	0.2	—	100±2	97±3
200	0.2	0.2	10^3	102±8	104±5
200	2.0	2.0	10^2	99±4	99±3
200	20.0	20.0	10	98±2	98±2
20	2.0	2.0	10	94±3	94±3
2.0	0.2	0.2	10	96±1	98±2
0.2	0.02	0.02	10	102±3	104±3

Preliminary experiments with nitration of pure Aroclors 1254 and 1260 showed that hepta- and octachlorobiphenyl constituents were much more difficult to nitrate if fuming nitric acid was not used. Aroclor 1254 constituents (mainly penta- and hexachlorobiphenyls) nitrated readily in concentrated nitric acid–sulfuric acid, but some interferences in the region of Mirex and photomirex remained on the GC trace. The reaction time (30 min) and temperature (70°C) chosen ensure essentially complete nitration of PCBs with 7 or fewer Cl/molecule.

The results of a recovery study where 0.02–20 μg Mirex and photomirex were nitrated along with varying amounts of 1:1 Aroclor 1254:1260 are given in Table 1. No detectable (<0.001 μg) interference was noted in blank runs. Analysis using an SP-2100 GC column indicated a slight interference equivalent to 0.004 μg Mirex from nitration of 200 μg PCB. This interference would give a 10% error at a PCB/Mirex ratio of 5×10^3, but was eliminated by choosing a more polar GC column. Recovery of Mirex and photomirex ranged from 94 to 100%, independent of the amount of PCB or Mirex present. The main effect of a high PCB/Mirex ratio was an increased standard deviation in the mean recovery.

The relevant chemical characteristics of the substrates used in the interlaboratory study are given in Table 2. Mirex was estimated directly

Table 2. Levels of PCB and estimated levels of Mirex before nitration (mean±SD of 3 replicates) in the substrates used for the interlaboratory study

Substrate	lipid, %	mg/kg (wet weight) PCB[a]	Mirex[b]	PCB/Mirex
Sediment	—	0.54±0.02	0.19±0.01	2.8
Eel	22.0	7.3 ±0.5	0.45±0.03	16
Carp	11.3	2.2 ±0.04	0.12±0.002	19
Gull egg	8.3	166 ±3	8.6 ±0.3	19

[a] Calculated as 1:1 Aroclor 1254:1260.
[b] Estimate based on peak with relative retention time 3.24 to DDE on a 1% SP-2100 GC column at 180°C (see Fig. 2).

from the PCB chromatograms to obtain a comparison with the post-nitration values. The apparent PCB/Mirex ratio was roughly constant in the biological substrates, but concentrations varied over a 75-fold range. Recoveries of Mirex and photomirex from substrates which contained no detectable Mirex before spiking averaged 93±7 for Mirex and 92±3% for photomirex at spiking levels similar to those found naturally in Lake Ontario substrates (0.1 ppm in sediment, 0.04 and 0.2 ppm in fish, 4 ppm in egg).

In Fig. 2, the "before" and "after" nitration chromatograms for each substrate are quantitatively compared. Mirex and photomirex are the only major constituents in the biological substrates to survive nitration; HCB, *t*-nonachlor, and small amounts of mono- and dihydromirex compounds constitute most of the minor peaks remaining in the chromatograms. HCB recovery is low (≤10%) because of volatilization rather than reaction with the acid. Replacement of all ring hydrogen atoms with chlorine protects against $-NO_2$ substitution in aromatic compounds.

The interlaboratory study results are presented in Table 3. The coefficient of variation (CV) of the 4 replicates ranged from 3 to 16% for Mirex and 2 to 16% for photomirex. The ratio of the grand mean "after nitration" values for Mirex to the "before nitration" values (Table 2) were 0.78, 0.68, 0.68, and 0.94 for sediment, eel, carp, and herring gull egg, respectively. Direct determination of Mirex from the PCB chromatogram did not, therefore, give a serious overestimate of the amount of Mirex present at these ratios of PCB/Mirex (20/1). However, a significant increase in this ratio, as is found, for example, in samples from the Great Lakes other than Lake Ontario, would make direct determination highly inaccurate.

The sediment extract posed some difficulties. Laboratory 2 found that a green color formed during nitration. This color extracted into the dichloromethane–hexane layer and hindered cleanup. The relatively high sulfur content of this sediment extract was probably responsible. Laboratories 3 and 4 also did not remove sulfur, however, and experienced no difficulties. Laboratory 1 removed sulfur by passing the

Table 3. Interlaboratory study results for the determination of Mirex and photomirex (mg/kg (wet weight) mean ± std dev. of 4 replicates) in naturally contaminated substrates from Lake Ontario

	Sediment		Carp		Eel		Gull egg	
Lab.	Mirex	Photo-mirex	Mirex	Photo-mirex	Mirex	Photo-mirex	Mirex	Photo-mirex
1	0.14 ±0.02	<0.01 —	0.088 ±0.007	0.028 ±0.003	0.34 ±0.05	0.11 ±0.02	8.2 ±0.6	2.8 ±0.2
2	n.a.[a] —	n.a. —	0.083 ±0.004	0.023 ±0.002	0.43 ±0.04	0.11 ±0.01	8.7 ±0.3	2.6 ±0.2
3	0.12 ±0.01	<0.01 —	0.061 ±0.003	0.022 ±0.001	0.31 ±0.04	0.14 ±0.01	6.8 ±0.3	2.8 ±0.1
4	0.17 ±0.02	0.002 ±0.001	0.079 ±0.003	0.018 ±0.002	0.33 ±0.02	0.08 ±0.01	8.7 ±0.3	2.2 ±0.1
Mean	0.14	<0.01	0.078	0.022	0.35	0.11	8.1	2.6
Std dev.	±0.03	—	±0.011	±0.004	±0.06	±0.02	±0.9	±0.3
CV, %	18	—	14	18	17	21	11	11
Repeatability CV,[b] %	—	—	6	9	11	11	5	5
Reproducibility CV,[b] %	—	—	16	20	18	23	12	12

[a] Not analyzed.
[b] See Ref. 20 for definitions of repeatability and reproducibility variance.

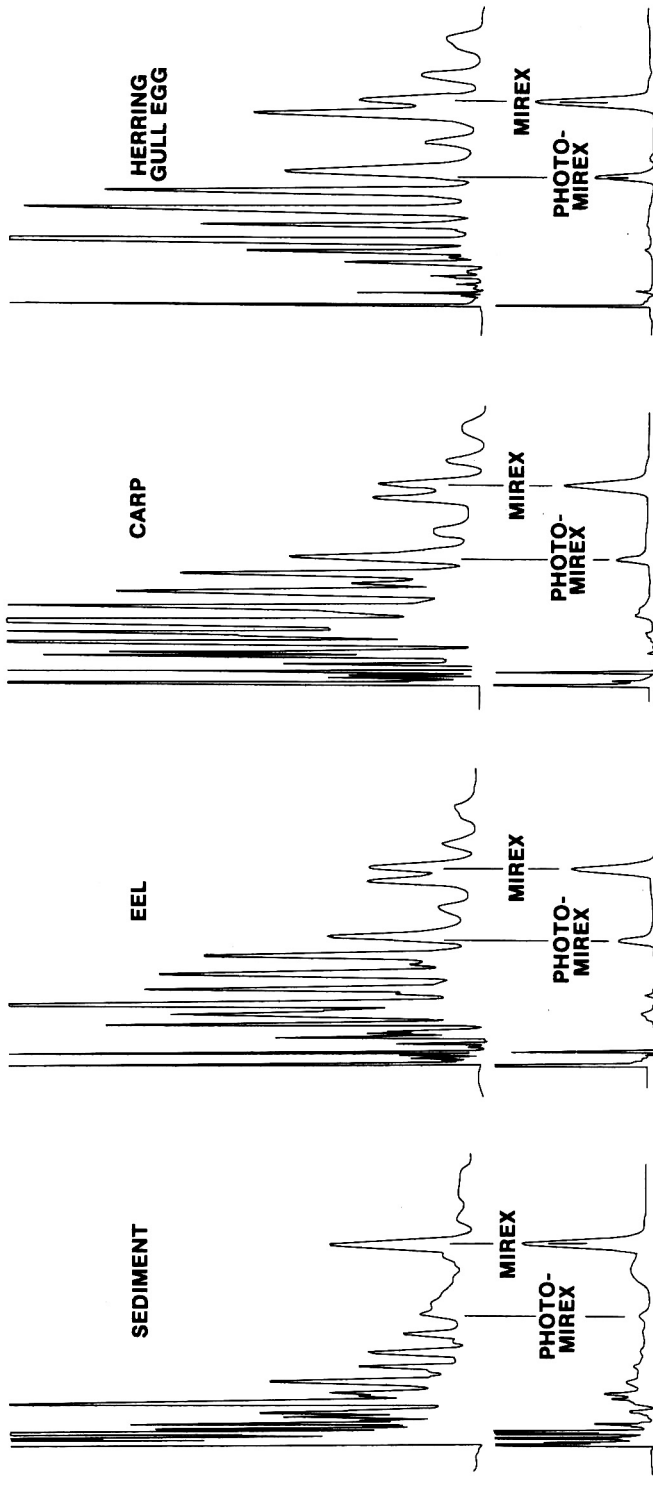

FIG. 2—Chromatograms of first fraction, Florisil cleanup (17), of Lake Ontario substrates before (top) and after (bottom) nitration (column: 180 cm × 2 mm id, 1% SP-2100 on 80–100 mesh Supelcoport, 180°C).

extract through a small column of copper powder before nitration. Although such a treatment may not always be necessary, it is suggested for all high sulfur (usually organic) sediments. Because of the missing data for sediment, further statistical analysis was confined to data for biological substrates.

Applying rank criteria for rejecting data (20) to the data for biological substrates, the Mirex values from Laboratory 3 and photomirex values from Laboratory 4 are just low enough to be rejected (total rank 3, minimum rank 4–5). However, because of the small number of laboratories, this criterion may not be valid and all data were retained for analysis of variance.

A range test for homogeneity of variation indicated lack of homogeneity at the 5% significance level. Homogeneity was not expected, because the CVs for Mirex and photomirex in carp and herring gull egg were similar, in spite of the 100-fold difference in concentration of these compounds. A one-sample analysis of variance (20) was therefore carried out on Mirex and photomirex data for each substrate. The results are summarized as CVs in Table 3. Interlaboratory differences were a significant ($p < 0.05$) source of variation in all cases except Mirex in eel, for which there was a relatively large repeatability CV. If the Mirex data from Laboratory 4 had been excluded according to the rank criterion, interlaboratory variance would not have been significant. Considering the range in concentrations, the small number of laboratories, and inexperience with the method, the reproducibility of the method is satisfactory.

Caution must be exercised in the following steps in order to obtain consistently good results from this method:

(*1*) Water must be added to the acid in such a fashion that Mirex and photomirex are not lost by steam distillation. (*2*) Lipids (from biological substrates) and sulfur (from sediments and soils) should be removed before nitration. (*3*) Fractionation into a less polar (Mirex, PCB, DDE) fraction and more polar (cyclodienes, DDT, HCH) fraction by column chromatography before nitration is recommended to reduce chances of interference from compounds which do not nitrate. (*4*) Linearity of response for Mirex and Mirex-related compounds in linearized electron capture detectors should not be assumed. This class of compounds has characteristically non-linear response in at least one brand of detector.

If these precautions are taken, the method is reliable for routine determination of Mirex and photomirex at levels ≥10 ppb in the presence of 1000-fold greater levels of PCB. The method is rapid, uses a minimum of unspecialized glassware, and requires only chemicals and materials commonly found in a residue laboratory.

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Gas-Liquid Chromatographic Determination of Bromacil Residues

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A simple, fast, and accurate method has been developed to determine residues of bromacil (5-bromo-3-*sec*-butyl-6-methyluracil) herbicide. Following sample extraction, filtration, and concentration, the herbicide is determined using a gas chromatograph equipped with a thermionic nitrogen/phosphorus detector. The method has been used to recover 0.04 ppm bromacil added to citrus, pineapple, soil, and water, and 0.1 ppm bromacil added to alfalfa hay.

Bromacil (5-bromo-3-*sec*-butyl-6-methyluracil) is a herbicide that is used for general weed control in noncrop areas, particularly against perennial grasses. It is also recommended for selective weed control in pineapple and citrus (1).

Presently, electron capture (2) and microcoulometric (3) gas-liquid chromatographic (GLC) methods are widely used for bromacil residue analysis. These methods involve extensive sample extraction and cleanup to determine a residue level as low as 0.04 ppm (2–4), and consequently the analysis is lengthy.

Recently, our laboratory has successfully developed a direct and accurate method for bromacil residue determination in various kinds of samples using GLC with a thermionic nitrogen/phosphorus detector. We believe that this method is faster and more accurate than existing methods.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Perkin-Elmer Sigma 2, equipped with thermionic nitrogen/phosphorus detector and 180 × 0.2 cm id glass column, packed with 1.5% OV-17/1.95% QF-1, on 100–120 mesh Chromosorb W (HP). Operating conditions: nitrogen flow rate 30 mL/min, make-up air flow rate 30 mL/min; hydrogen pressure 10 psi, air pressure 26 psi; bead adjust 5.1; temperatures (°C)—column 250 (230 for pineapple, 220 for alfalfa hay), detector 300, injection port 280 (250 for alfalfa hay); range 1, attenuation 8, auto zero on; Perkin-Elmer recorder 023, chart speed 1 cm/min.

(b) *Food chopper.*—Model 8181-D (Hobart Mfg. Co., Troy, OH 45374).

(c) *Homogenizer.*—Omni-Mixer (Du Pont Co., Bio Medical Division, Newton, CT 06410).

(d) *Wiley mill.*—Motor-driven. Intermediate model, equipped with 20-mesh metal sieve (Arthur H. Thomas Co., Vine St, Philadelphia, PA 19105).

(e) *Water bath.*—Model WBT-100, equipped with thermostat (Barnstead Still and Sterilizer Co., 225 Rivermoor St, Boston, MA 02132).

(f) *Solvent.*—Ethyl acetate AR grade.

(g) *Bromacil standard solutions.*—(1) *Stock solution.*—1 mg bromacil/mL ethyl acetate. (2) *Intermediate solution.*—1 μg bromacil/mL ethyl acetate. (3) *Working solution.*—0.2 μg bromacil/mL ethyl acetate.

Preparation of Sample

Citrus and pineapple.—Finely chop fruit in Hobart food chopper. Weigh 100 g sample of well chopped fruit into 1 pt Mason jar. Accurately add 100 mL ethyl acetate to jar and blend 3 min with Omni-Mixer. Let mixture settle until ethyl acetate separates from fruit juice and solids. Carefully pour top ethyl acetate layer through sharkskin filter paper; collect filtrate in flask. Pipet 10 mL filtrate into 100 mL beaker, and evaporate just to dryness on 60°C water bath with gentle air stream. Accurately pipet 2 mL ethyl acetate into beaker and rotate gently. Solution is ready for injection into gas chromatograph. A sample size of 2–5 μL is usually recommended for injection; however, the exact injection volume depends on the residue concentration in the final solution.

Alfalfa hay.—Grind ca 35 g dry alfalfa hay through Wiley mill with 20-mesh sieve; weigh exactly 20 g ground hay into 1 pt Mason jar. Pour about 100 mL water into jar. The dried hay will become saturated in about 30 min. Rehydration is necessary to prevent ethyl acetate from being completely absorbed by hay. Accurately measure 50 mL ethyl acetate into jar, then homogenize 3 min with Omni-Mixer. Let mixture settle, then filter top ethyl acetate layer through sharkskin paper into flask. Proceed as described above for citrus and pineapple sample preparation.

Soil.—Weigh 25 g well mixed soil into 1 pt Mason jar. Measure 25 mL ethyl acetate into jar and mix contents well. Filter ethyl acetate layer through sharkskin paper and proceed as described above for citrus and pineapple sample preparation.

Water.—Measure 100 mL water into 1 pt Mason jar. Pour 100 mL ethyl acetate into jar and mix contents well. Filter top ethyl acetate layer through sharkskin paper and proceed as described above for citrus and pineapple sample preparation.

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Table 1. Recovery of bromacil added to several sample types

Sample	Added, ppm	Found, ppm	Rec.,[a] %	Rel. dev.,[b] %	Rel. error,[c] %
Citrus					
Orange	0.04	0.041	103	13.8	1.5
Grapefruit	0.04	0.035	88	11.1	12.8
Lemon	0.04	0.037	93	6.6	6.8
Pineapple	0.04	0.042	105	9.6	4.3
Alfalfa hay	0.10	0.096	96	4.3	4.2
Soil	0.04	0.041	103	5.2	3.3
Water	0.04	0.044	110	3.0	9.8

[a] Each value is the average of 3 replicates.
[b] Relative deviation, % = standard deviation/mean × 100.
[c] Relative error, % = (observable value − actual value)/actual value × 100.

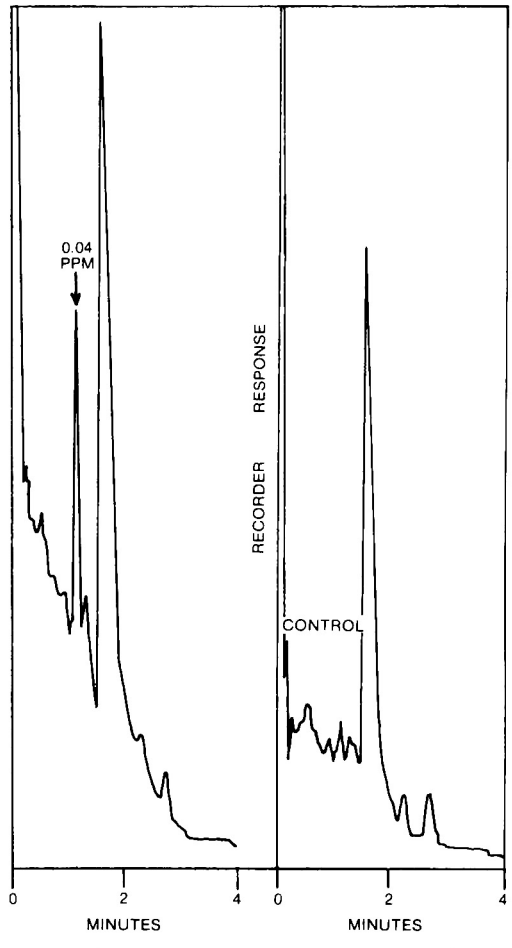

FIG. 1—Chromatogram of recovery of bromacil from orange.

Results and Discussion

The thermionic nitrogen/phosphorus detector has excellent linearity for a wide range of bromacil concentrations from 0.2 to 1.2 ng. The range of peak heights was from 5 to 40% deflection on a 100% full scale chart, and the noise level was less than 1%. The peak shapes were slightly tailed; consequently, the peak heights were measured from the baseline at the rising point to the peak top in all circumstances.

The results of citrus, pineapple, alfalfa, soil, and water analyses are shown in Table 1. Recoveries were 88% or more for all samples tested. The percentage values for relative deviation and relative error were fairly good considering the small sample numbers, especially since the bromacil residue level was as low as 0.04 ppm. The values for both relative deviation and relative error could be improved substantially if the bromacil residue level was increased to 0.1 ppm or more. This has been demonstrated in the case of alfalfa hay.

The 250°C column temperature was adequate for most samples. Chromatograms of orange, soil, and water are shown in Figs 1–3. An unknown compound in pineapple with a retention time close to that of bromacil at 250°C was easily separated from bromacil after the column temperature was reduced to 230°C (Fig. 4).

Low-boiling volatile compounds in alfalfa hay contributed many early peaks on the chromatogram and interfered with the appearance

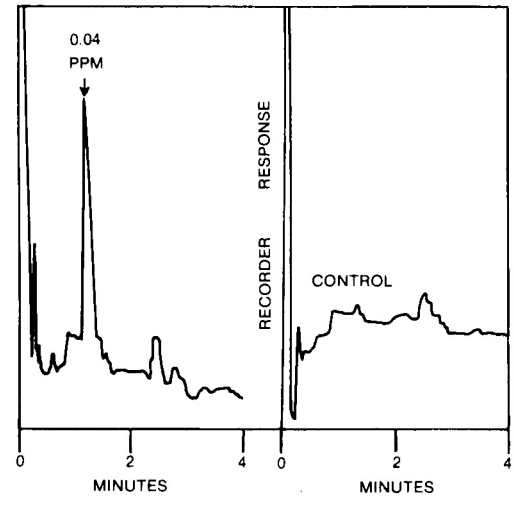

FIG. 2—Chromatogram of recovery of bromacil from soil.

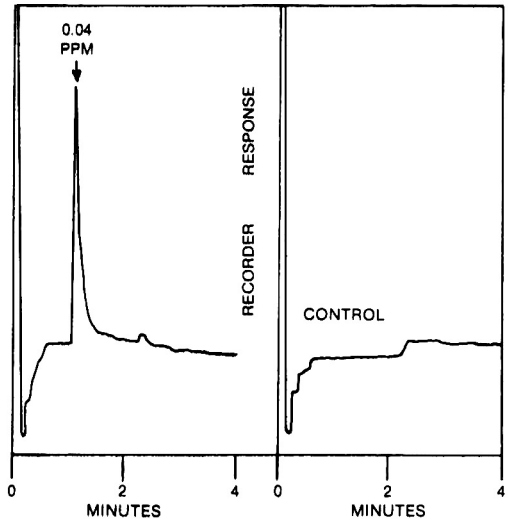

FIG. 3—Chromatogram of recovery of bromacil from water.

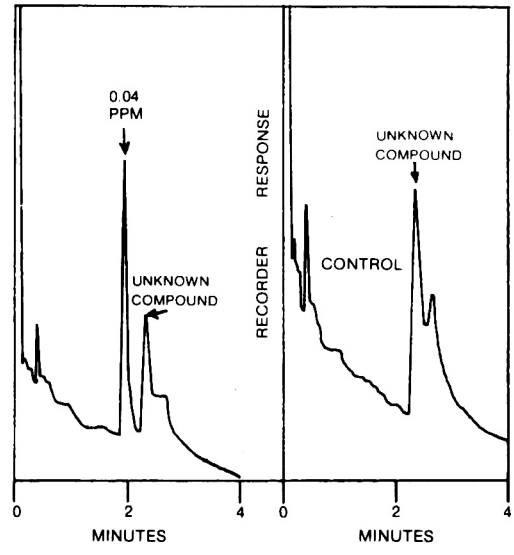

FIG. 4—Chromatogram of recovery of bromacil from pineapple.

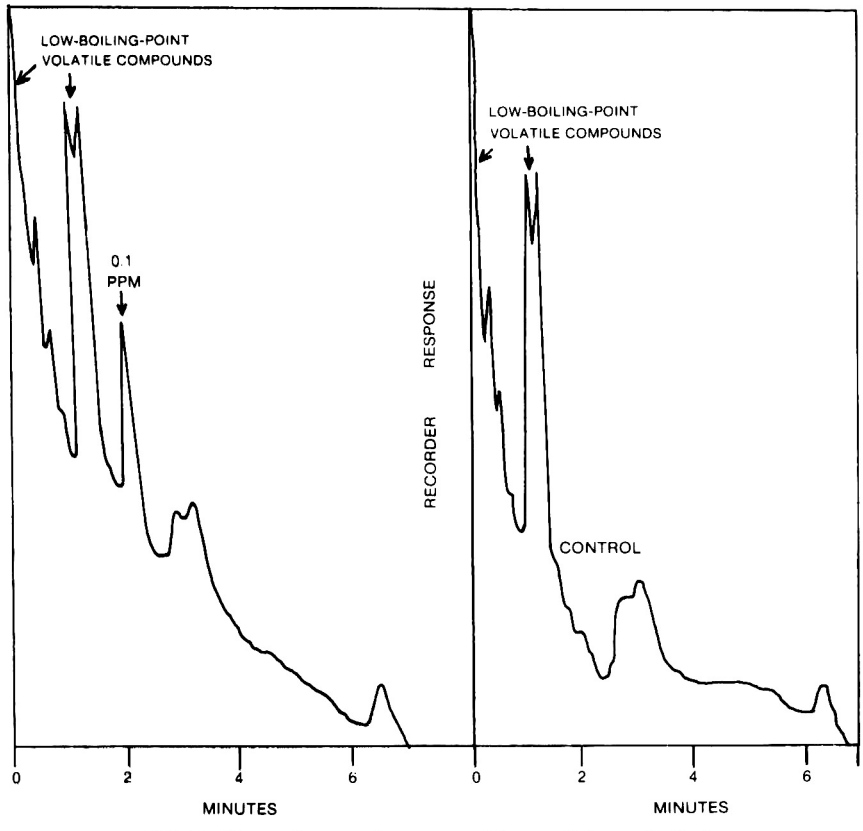

FIG. 5—Chromatogram of recovery of bromacil from alfalfa hay.

of bromacil. Therefore, the injector and column temperatures were reduced to 250 and 220°C, respectively, to prolong the bromacil retention time and separate the bromacil from the interfering compounds. The chromatogram is presented in Fig. 5.

We used a composite soil sample, a typical Southern California sandy loam soil, from Imperial, Riverside, and San Bernardino counties, California. We believe that our method can be applied to various types of soil except clay; the method could be modified by using heat-reflux extraction to enhance ethyl acetate penetration power for clay-type soils. In addition, we recommend performing a recovery study before testing any soil sample as part of the quality control.

The greatest advantage in this method is the simplicity of sample preparation without any sacrifice of accuracy. In fact, we obtained results instantly by injecting unconcentrated filtrate immediately following the ethyl acetate extraction for bromacil sample concentrations of 1.0 ppm or more for alfalfa hay and 0.1 ppm or more for all others. The fundamental reason for the simplicity of the method is the excellent sensitivity and selectivity of the thermionic nitrogen/phosphorus detector in response to bromacil herbicide. Its unique capability has given us sufficient justification to eliminate the lengthy cleanup procedure necessary in other methods (2–6). We believe that this short and accurate method for bromacil residue analysis is worthy of further study.

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High Performance Liquid Chromatographic Determination of Bendiocarb on Wool

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A high performance liquid chromotographic method is presented for determining bendiocarb (2,2-dimethyl-1,3-benzodioxol-4-yl methylcarbamate) on wool. Bendiocarb is extracted from wool with methanol containing methyl benzoate as internal standard, eluted through a Zorbax ODS column with methanol-water (55 + 45), and detected with a UV detector at 280 nm. The method can be used to determine bendiocarb at 0.001–0.02% by weight. The limit of detection is 0.0004%, or 4 ppm. At 4 analyses each, recovery at 0.013% was 101%, standard deviation 2.8%; at 0.003%, recovery was 96%, standard deviation 5.6%; at 0.001%, recovery was 103%, standard deviation 2.9%.

Bendiocarb (2,2-dimethyl-1,3-benzodioxol-4-yl methylcarbamate), also known as Fisons NC 6897 and Ficam®, is a broad spectrum insecticide (1, 2). It has also shown promise as a protectant against insect damage on woolen fabrics.

Bendiocarb cannot be analyzed by gas chromatography because it decomposes at high temperatures, but it can be analyzed by high performance liquid chromatography (HPLC) at relatively low or ambient temperatures. Therefore, a method for the determination of bendiocarb on wool was developed and is reported here.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—DuPont Model 820 with 25 cm × 2.1 mm stainless steel column packed with Zorbax ODS (E. I. du Pont de Nemours & Co., Inc., Wilmington, DE 19898). Operating conditions: column temperature, ambient; column pressure, 1500 psig; flow rate, 0.25 mL/min.

(b) *Detector.*—Variable wavelength at 280 nm, Laboratory Data Control Spectro Monitor II (Laboratory Data Control Division, Milton Roy Co., Riviera Beach, FL 33404).

(c) *Liquid sample valve.*—3000 psig rated (Altech Associates, Arlington Heights, IL 60004).

(d) *Mobile phase.*—Methanol–water (55 + 45 v/v).

(e) *Methanol.*—Pesticide grade (Fisher Scientific Co., Pittsburgh, PA 15219).

(f) *Standard.*—Bendiocarb, 99% (Fisons Corp., Bedford, MA 01730).

(g) *Methyl benzoate.*—Aldrich Chemical Co., Milwaukee, WI 53233.

(h) *Stock solutions.*—*(1)* Internal standard: 0.005% methyl benzoate. Dissolve 50 mg methyl benzoate in methanol to make 1 L. (2) Bendiocarb: Dissolve 100 mg bendiocarb in methanol to make 100 mL and dilute 1:100 in methanol to make 0.0010% stock solution for response calculation and recovery determination.

Standard Curve

Mix known volumes of 0.001% bendiocarb with 5 mL 0.005% methyl benzoate internal standard solution and dilute to 100 mL with methanol. Remove 1 mL, dilute to 5 mL with water, mix thoroughly, and inject into liquid chromatograph, using sample valve with 1 mL loop. Measure peak heights of methyl benzoate and bendiocarb. Plot peak height ratio of bendiocarb to methyl benzoate vs. known concentration of bendiocarb.

Bendiocarb on Wool

Weigh 3 × 4 in. (7.6 × 10.2 cm) swatch of wool and cut into small pieces. Place in 250 mL Erlenmeyer flask, add 95 mL methanol and 5.00 mL 0.005% methyl benzoate, and agitate 3 hr. Remove 1 mL, dilute to 5 mL with water, and inject 1 mL into liquid chromatograph with sampling valve. Measure peak heights of methyl benzoate and bendiocarb and calculate their ratio. Determine amount of bendiocarb from standard curve or calculate per cent from following equation:

$$\text{Bendiocarb, \%} = (w' \times p' \times PH \times 100)/(w \times PH' \times RF)$$

where w and w' are weights of sample and methyl benzoate, respectively; p' is per cent purity of methyl benzoate; PH and PH' are peak heights of bendiocarb and methyl benzoate, respectively; and RF is response factor calculated from known sample as follows:

$$RF = (w' \times p' \times PH)/(w \times p \times PH')$$

where p is per cent purity of bendiocarb standard.

Mention of a pesticide or commercial or proprietary product in this paper does not constitute a recommendation of this product by the U.S. Department of Agriculture.

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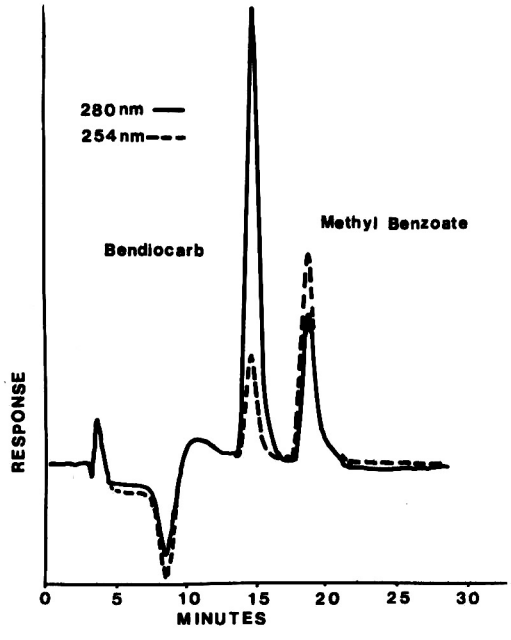

FIG. 1—Chromatograms of 0.619 μg bendiocarb and 0.50 μg methyl benzoate with UV detector at 280 and 254 nm, 0.08 AUFS.

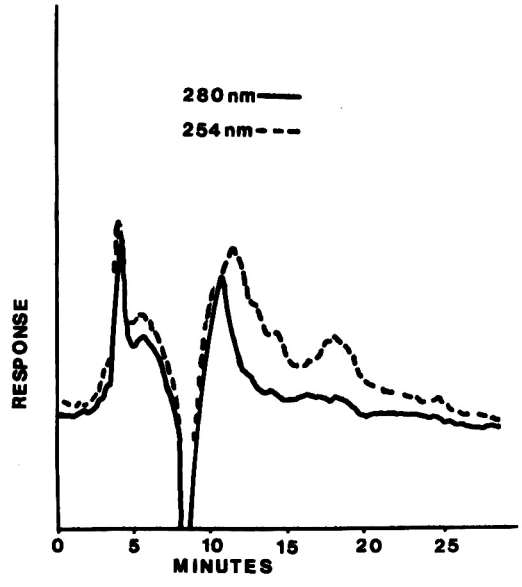

FIG. 2—Chromatograms of untreated wool extract with UV detector at 280 and 254 nm, 0.04 AUFS.

Per cent purity of methyl benzoate may be omitted if same source is used for *RF* and unknown determinations.

Results and Discussion

The method can be used to determine 0.001–0.02% by wt of bendiocarb on wool, which covers the range being studied as a protectant against fabric insects. Based on 4 analyses each, the recovery of bendiocarb at approximately 0.013% was 101% with a standard deviation of 2.8%; at 0.003%, recovery was 96% with a standard deviation of 5.6%; and at 0.001%, recovery was 103% with a standard deviation of 2.9%. The limit of detection under the conditions used is approximately 0.0004%, or 4 ppm.

Figure 1 is a sample chromatogram. The response of bendiocarb is greater at 280 nm and also below 254 nm than at the 254 nm mercury vapor line. The responses of interfering compounds extracted from wool also are enhanced at wavelengths below 254 nm. Chromatograms of wool extract at 254 and 280 nm (Fig. 2) show that the response of interferences was less at 280 nm than at 254 nm.

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Gas-Liquid Chromatographic Determination of Sodium Fluoroacetate (Compound 1080)

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An analytical method is described for the determination of Compound 1080 (sodium fluoroacetate) residues in 1–10 g tissue. Sample extracts of tissues are cleaned up with silica gel, and Compound 1080 (as fluoroacetic acid) is separated by a micro-distillation procedure. The fluoroacetic acid in the distillate is derivatized with pentafluorobenzyl bromide to form pentafluorobenzyl fluoroacetate which is measured by electron capture gas-liquid chromatography. Recoveries of sodium fluoroacetate from fortified tissue samples averaged about 25%. Despite the limited recoveries, results were quite reproducible, and levels as low at 2 ppm were determined in fortified 1 g samples, and 0.2 ppm in 10 g samples. The method is relatively simple and has been used routinely in our laboratory for the analysis of various types of samples such as grain, and tissues from birds, rodents, and larger animals.

Sodium fluoroacetate, commonly called Compound 1080, has been widely used in the past for the control of rodents and certain predators. However, because of its extreme toxicity and lack of adequate data to determine safety, it has been restricted by law to approved experimental uses on nonpublic lands. Our laboratory has been involved with various studies to evaluate the efficacy of Compound 1080 as an animal damage control agent and the environmental hazards associated with its use. These studies required the determination of 1080 residues in various types of tissue materials including small samples of tissues from rodents. Available analytical methods include the use of enzyme inhibition (1), citrate formation (2), colorimetry of fluoroacetic acid (3, 4), total fluorine by colorimetry (4, 5) or ion-selective electrode (6, 7), and gas-liquid chromatography (GLC) (8–12). However, these methods lack specificity, are lengthy, or require a large quantity of sample for adequate sensitivity. The enzymatic, citrate, and colorimetric methods are only qualitative, whereas total fluorine methods, although quantitative, are not specific. GLC methods specify flame ionization or mass spectrometric detection to measure the methyl or ethyl ester of fluoroacetic acid. Because of the relatively low molecular weight of these esters, quantities below 50–100 ng cannot be easily measured by flame ionization detectors. Relatively large samples (50 to 100 g) need to be extracted, and the final sample solution must be concentrated before analysis by GLC. Background interferences are frequently encountered if good sample cleanup techniques are not used.

Pentafluorobenzyl bromide (PFBB) has been used by workers for the GLC determination of organic acids and phenolic type compounds (13–18). We have found that it reacts readily with fluoroacetic acid to form pentafluorobenzyl (PFB) fluoroacetate, which is detectable at picogram levels by electron capture (EC) techniques. In the method described here, the free acid of Compound 1080 is separated from the sample by silica gel cleanup and micro-distillation procedures, derivatized with PFBB, and measured by GC with an EC detector.

METHOD

Reagents and Apparatus

(a) *Solvents.*—Distilled-in-Glass® grade (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

(b) *Silica gel.*—(Davison Chemical, Baltimore, MD 21226), Grade 950, 60–200 mesh, used as received.

(c) *Pentafluorobenzyl bromide, α-bromo-2,3,4,5,6-pentafluorotoluene.*—Aldrich Chemical Co., Milwaukee, WI 53233), 10% solution in hexane. (Caution: Reagent is a strong lachrymator.)

(d) *Sodium fluoroacetate.*—(Sigma Chemicals Co., St. Louis, MO 63178), 1000 μg/mL acetone (10% v/v water).

(e) *Fluoroacetic acid.* — (Columbia Organic Chemicals Inc., Columbia, SC 29209), 1000 μg/mL acetone.

(f) *Micro-distillation apparatus.*—Consists of two screw-cap culture tubes (15 × 110 mm) with Bakelite caps, sample tube, and receiver tube, connected by Teflon tubing (3 mm × ca 35 cm). A shorter Teflon tubing (2 mm × ca 5 cm) connects sample tube to source of nitrogen gas. Cap for sample tube is fitted with liner consisting of ⅜ in. diameter Teflon-lined gas chromatograph septum. Holes in septum are bored smaller than diameter of tubings to ensure gas-tight fit.

Use of trade names does not imply endorsement of commercial products by the United States Government.

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(g) *Gas chromatograph.*—Tracor Model 560, equipped with ^{63}Ni detector and linearizer (standing current display on 1 mV recorder, 1×10^{-9} amp full scale); and two 183 cm × 2 mm id glass columns, one packed with 3% DC-200/12,500 (100–120 mesh Chromosorb W, DMCS), the other packed with 1:1 mixture of 3% DC-200 and 5% QF-1 (100–120 mesh Chromosorb W, DMCS). Operating conditions: Column 105°C, inlet 125°C, detector 350°C, carrier gas (10% methane/argon)—DC-200 column 20 mL/min, mixed column 40 mL/min.

(h) *Gas chromatograph-mass spectrometer.*—Varian Model 204 gas chromatograph interfaced with Watson-Biemann separator to Nuclide 1290-G mass spectrometer; ionizing potential 70 eV; accelerating voltage 5 kV; source pressure 2×10^{-5} torr.

Procedure

Tissue samples.—Weigh 1–10 g homogeneously ground sample into 20 × 120 mm screw-cap culture tube, add acetone (5 mL for 1–5 g samples and 10 mL for 10 g samples), and mix in ultrasonic bath ≥15 min. Centrifuge 3 min or until upper layer is clear; then decant supernate into tared 50 mL beaker. Repeat extraction of sample with same amount of acetone, centrifuge, and transfer supernate to 50 mL beaker. Reduce volume of solution in beaker to ca 5 mL with stream of nitrogen or air; then add exactly 3 g silica gel to beaker and stir. Place beaker on hot plate (surface temperature near 120°C), and heat, with occasional stirring, until acetone and any water present evaporate and net weight of silica gel is reached. Transfer dried silica gel to a 20 × 120 mm screw-cap culture tube. Add 10 mL acetone, cap, mix in ultrasonic bath 5 min, centrifuge until clear, and discard acetone; repeat wash once with 10 mL acetone. Add 5 mL acetone, 5 drops (medicine dropper) of concentrated H_3PO_4, shake 30 sec, mix in ultrasonic bath 5 min, centrifuge, and transfer supernate to 15 × 110 mm screw-cap tube. Repeat extraction of silica gel with 5 mL acetone. Place tube with sample solution in water bath maintained at 55–60°C and reduce volume to ca 2 mL with stream of nitrogen; then proceed to micro-distillation procedure.

Bait samples.—Place 1 g dry, homogeneously ground sample in 15 × 110 mm screw-cap culture tube, add 5 mL acetone and 5 drops of concentrated H_3PO_4, and mix in ultrasonic bath 10 min. Centrifuge 3 min, and transfer extract to second screw-cap tube; repeat extraction of sample once with 5 mL acetone. Place second tube in water bath (55–60°C) and reduce volume of extract solution to ca 2 mL with stream of nitrogen; then proceed to micro-distillation procedure.

Micro-distillation.—Connect tube containing sample solution to another 15 × 110 mm tube containing 5 mL acetone, as described under *Reagents and Apparatus.* Position delivery end of Teflon tubing under surface of acetone and near bottom of tube. Connect shorter Teflon tubing to source of nitrogen gas and adjust flow rate to ca 100 mL/min. Place entire sample tube in tube heater (Kontes, or equivalent) heated at 95°C, and receiver tube in ice-water bath (with multiple tube heater, several distillations can be made simultaneously). Distill ≥30 min or until all acetone has distilled and no condensate remains in connecting Teflon tubing. Avoid bumping or splattering during distillation to prevent H_3PO_4 from being carried over into distillate, because presence of acid in distillate will seriously interfere in subsequent derivatization step. When distillation is complete, remove tubes from heater and bath; let heated tube cool, remove cap, and wash Teflon tubing with 1 mL acetone from hypodermic syringe, collecting washing in receiver tube. If distillate is believed to contain high concentration of fluoroacetic acid (such as in the analysis of bait samples), use small aliquot containing <50 μg fluoroacetic acid for derivatization procedure.

Derivatization and gas chromatographic analysis.—To distillate in receiver tube, add 0.2 g K_2CO_3 and 20 μL 10% PFBB derivatizing solution, cap, mix, and heat in tube heater (or equivalent) at 50°C for 30 min. Let tube cool, and dilute with acetone to precalibrated 10 mL mark.

Prepare GC reference solutions by derivatizing appropriate amounts of 1000 μg sodium fluoroacetate/mL solution under same conditions as sample. An injection of 1 ng (as sodium fluoroacetate) of derivative should result in full scale response at attenuation of 128. Analyze reference and sample solutions on both chromatographic columns for qualitative confirmation. Calculate ppm Compound 1080 with column that allows better quantitation using baseline extrapolation and peak height.

Results and Discussion

Various procedures for PFBB derivatization have been described by other workers (13–18). They include the use of acetone or alcohol as the solvent, and alcoholic KOH or aqueous K_2CO_3 as the base. We found that derivatization proceeded best when acetone and dry K_2CO_3, instead of an aqueous solution, was used. Alcohol was not satisfactory and the presence of water significantly reduced the yield of the derivative. The time required for complete esterification of 10 μg fluoroacetic acid varied from 40 min at 30°C to only 10 min at 95°C (Table 1). At 50°C, derivatization of 1–100 μg fluoroacetic acid was complete within 20 min, but only partial conversion occurred

Table 1. Derivatization (as per cent of completion) of fluoroacetic acid with PFBB

	μg Fluoroacetic acid and temp.									
Deriv. time, min	10, 30°C	10, 40°C	1, 50°C	5, 50°C	10, 50°C	50, 50°C	100, 50°C	200, 50°C	300, 50°C	10, 95°C
10	—	80	—	—	90	—	—	—	—	100
15	—	96	—	—	98	—	—	55	—	
20	77	100	100	100	100	100	100	61	29	
30	86							65	—	
40	100							—	—	
60								74	—	
120								100	—	

with larger amounts; at least 2 hr was required for complete esterification of 200 μg at 50°C.

Although derivatization proceeded more rapidly at 95°C, undesirable esterification of other naturally occurring acids in the sample took place to a greater extent and complicated quantitation of the PFB fluoroacetate peak in the GLC analysis. To minimize interfering peaks, derivatization of samples was carried out at 50°C for 30 min. For the analysis of samples containing high concentrations of Compound 1080 (such as bait), only a portion of the distillate from the sample extract solution was derivatized to maintain a sufficient excess concentration of PFBB.

The PFB fluoroacetate is measurable at picogram levels by EC detection. An injection of 50 pg (as sodium fluoroacetate) generally resulted in a response of about 5% of full scale at an electrometer attenuation of 64 (Fig. 1). Although smaller quantities of the ester can be measured, the detectability and the recovery of Compound 1080 depend on the nature of the sample and the extent of interference from the sample matrix in the GLC analysis. This is shown by the differences in gas chromatograms obtained for the analysis of a 5 g sample of coyote liver fortified with 0.5 ppm Compound 1080 (Fig. 2), and for a 10 g sample of muscle tissue fortified with 0.2 ppm (Fig. 3). The large peaks just preceding and following the fluoroacetate peak vary in size with type of tissue and freshness of sample, and interfere to a greater extent in liver than in other tissues and in decayed samples than in fresh samples. The interference from these peaks is considerably smaller at higher levels of Compound 1080, as shown by the chromatograms from a liver sample fortified with 2 ppm (Fig. 4). Figure 5 shows gas chromatograms from the analysis of a 1 g sample of gastrointestinal tract that was removed from a ground squirrel poisoned in a field study, and found to contain 2 ppm Compound 1080 after correction for recovery.

GLC separation of the fluoroacetate peak may vary with different instruments and sample types. Therefore, conditions such as the relative amount of the stationary phases in the mixed column, the oven temperature, and carrier gas flow should be optimized to obtain best results.

In addition to the use of two GLC columns for greater specificity, GLC-mass spectrometry (MS) may be used for the confirmation of 1080 in some samples. Figure 6 shows the mass spectrum obtained by scanning the total ion

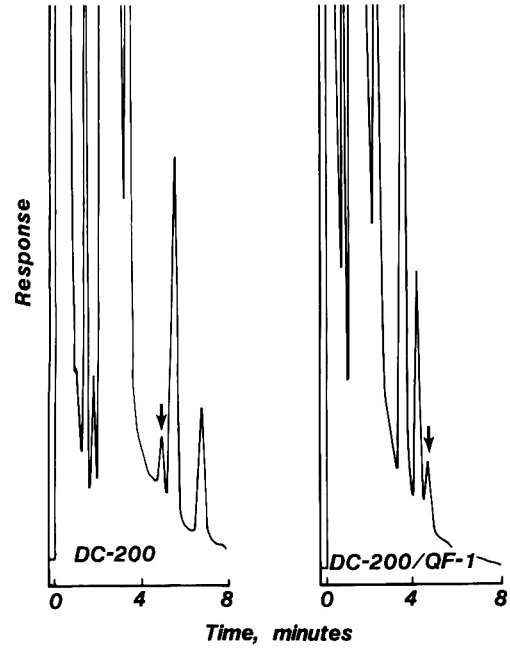

FIG. 1—Chromatograms of PFB fluoroacetate reference, 1 μL 0.05 ng/μL (as sodium fluoroacetate). Attenuation: 64. Arrow indicates PFB fluoroacetate peak.

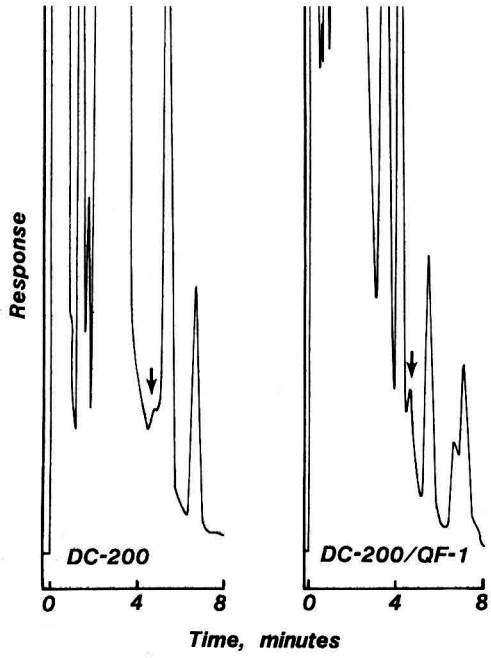

FIG. 2—Chromatograms of 5 g sample of coyote liver tissue fortified with 0.5 ppm Compound 1080; 1 μL injection (sample equivalence 0.5 mg/μL). Attenuation: 64. Arrow indicates PFB fluoroacetate peak. Not shown are other large peaks that emerge between 8 and 18 min on both columns.

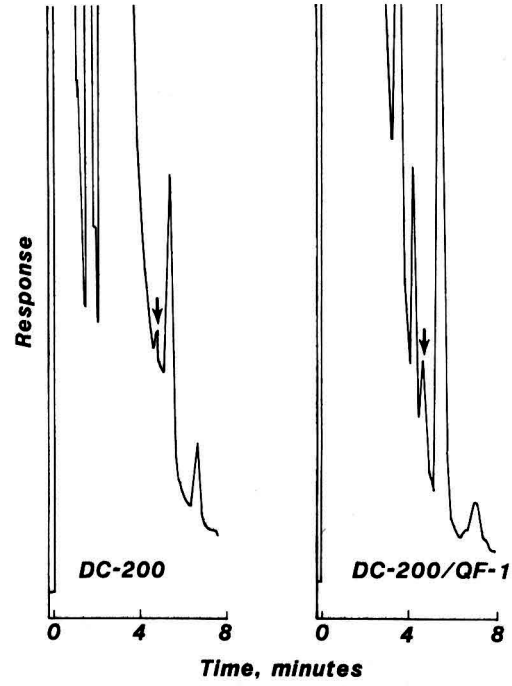

FIG. 3—Chromatograms of 10 g sample of coyote muscle tissue fortified with 0.1 ppm Compound 1080; 1 μL injection (sample equivalence 1.0 mg/μL). Attenuation: 32. Arrow indicates PFB fluoroacetate peak. Not shown are other large peaks that emerge between 8 and 18 min on both columns.

monitor peak at the retention time of the PFB ester (35 ng as fluoroacetic acid). The spectrum shows a strong molecular ion at m/e 258, the relative intensity of which is about 20% of the base peak $[C_6F_5CH_2]^+$ at m/e 181. Other significant peaks include the m/e 61 $[CH_2FCO]^+$ ion, and the m/e 33 $[CH_2F]^+$ ion.

Sample sizes of 1–5 g were suitable for the analysis of most tissues, especially those containing appreciable amounts of water such as blood and stomach content. With samples such as muscle tissues that contain less water and other interferences, 10 g material could be satisfactorily analyzed. Average recoveries of Compound 1080 from fortified 1 g samples of stomach content from a ground squirrel ranged from 14.5% at 2 ppm to 26% at the 10 ppm level (Table 2). Recoveries from 5 g samples of various tissues from a coyote averaged about 26% (Table 3). Liver samples, which were more difficult to analyze because of greater interferences, yielded lower recoveries of 8% at the 0.5 ppm level. The detection limit of the method was about 0.5 ppm for 5 g samples and about 0.2 ppm for 10 g samples. With 1 g samples of bait (Table 4), recoveries averaged 18% at the 1–10 ppm levels, and 38% at the 100–800 ppm levels. Overall recoveries were generally reproducible, and the average coefficient of variation was about 17% for ground squirrel stomach contents, 8% for coyote tissues, and 12% for bait.

The generally low recoveries are attributed to cumulative losses in the silica gel and microdistillation procedures, as indicated by comparable average recoveries of 26% for the fortified tissue samples (Table 4), and 29% for the combined silica gel and distillation procedures (Table 5). As shown in Table 5, recoveries (for 1 and 10 μg fluoroacetic acid) averaged 47 and 63%, respectively, for the distillation and silica gel steps. The silica gel, in addition to serving as a sample cleanup step, facilitates removal of water from the sample

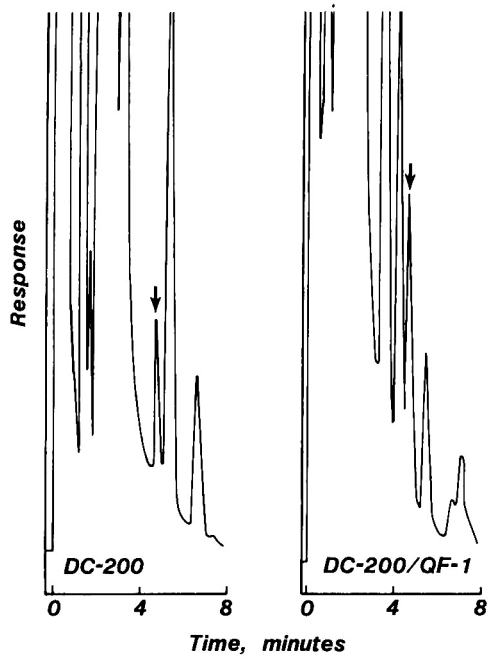

FIG. 4—Chromatograms of 5 g sample of coyote liver tissue fortified with 2 ppm Compound 1080; 1 μL injection (sample equivalence 0.5 mg/μL). Attenuation: 64. Arrow indicates PFB fluoroacetate peak. Not shown are other large peaks that emerge between 8 and 18 min on both columns.

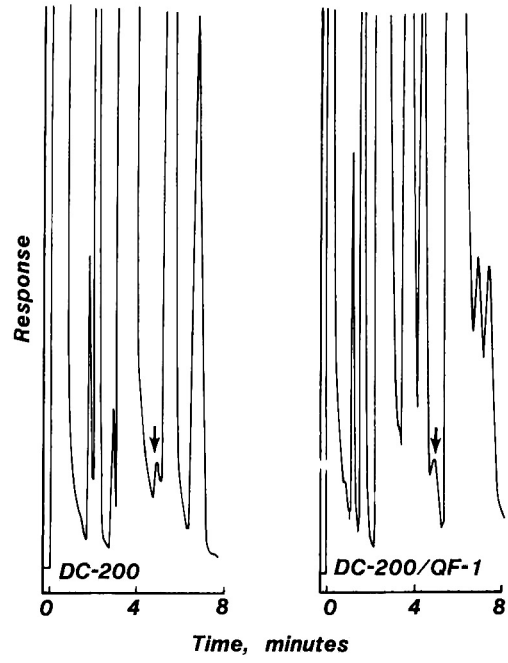

FIG. 5—Chromatograms of 1 g sample of gastrointestinal tract from a ground squirrel; 1 μL injection (sample equivalence 0.1 mg/μL). Attenuation: 128. Arrow indicates PFB fluoroacetate peak. Not shown are other large peaks that emerge between 8 and 18 min on both columns.

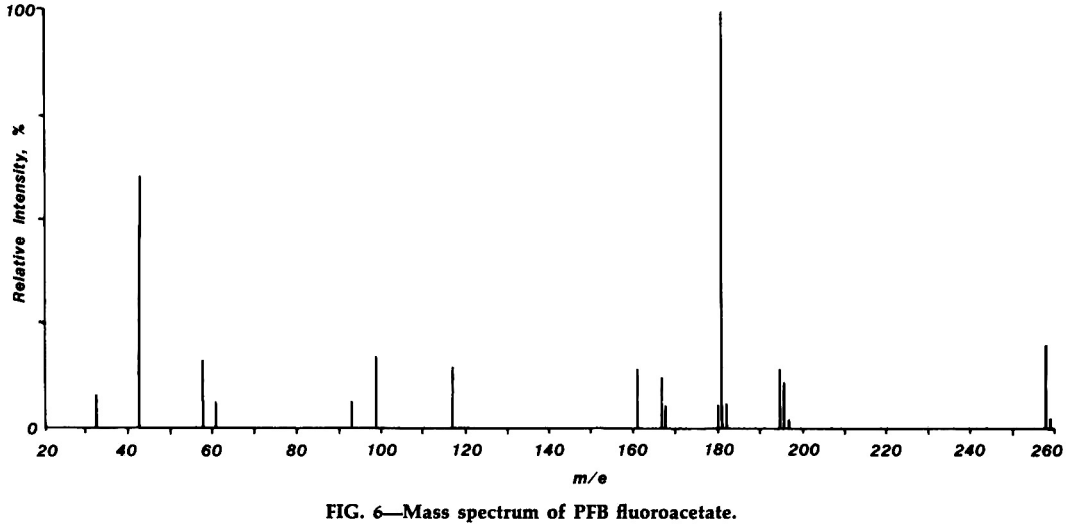

FIG. 6—Mass spectrum of PFB fluoroacetate.

Table 2. Recovery of Compound 1080 from fortified samples (1 g wet wt) of stomach content from a ground squirrel

Added, ppm	Found, ppm	Recovery, %	Av. rec., % (std dev.)
2	0.24	12	
	0.24	12	14.5 (3.0)
	0.36	18	
	0.32	16	
5	0.80	16	
	1.1	22	23 (7.0)
	1.5	30	
10	2.3	23	
	2.8	28	26 (2.6)
	2.7	27	

Table 3. Recovery of Compound 1080 from fortified samples of coyote tissues

Sample	Wet wt, g	Added, ppm	Found, ppm	Av. rec., %
Blood	5	0.5	0.12	22
			0.10	
Heart	5	0.5	0.14	30
			0.16	
Kidney	5	0.5	0.12	24
			0.12	
		1.0	0.25	25
			0.26	
Liver	5	0.5	0.04	8
			0.04	
		1.0	0.25	26
			0.27	
Muscle	5	0.5	0.15	26
			0.11	
	10	0.2	0.06	27
			0.05	

extract. Water and other co-extracted substances less adsorbed than fluoroacetic acid are volatilized from the silica gel with heat. Fluoroacetic acid, which is retained on the silica gel even when heated to 120°C, is subsequently desorbed with H_3PO_4 and acetone. The microdistillation separates fluoroacetic acid from H_3PO_4 and other nonvolatile substances and serves as an additional cleanup step. The low recoveries in the distillation may result partially from loss of volatilized fluoroacetic acid. Addition of K_2CO_3 to the acetone in the receiver tube did not improve recoveries.

Table 6 shows results obtained for the analysis of bait and various types of tissue samples from animals collected in an experimental field study to evaluate Compound 1080 as a rodent control agent. Sample types consisted mostly of stomach contents from small and large mammals, and internal organs and crop and gizzard contents from birds. The samples, which were received unidentified and randomly numbered to eliminate bias in the analysis, included tissues from animals collected before and after treatment of a field with Compound 1080 bait. Of 45 pretreatment tissue samples and 4 untreated bait samples analyzed, no false positive result was obtained. Residues of Compound 1080 were found in 41 of 111 post-treatment tissue samples and 19 of 22 treated bait samples. Many of the tissue samples were from animals that were decayed or less than fresh and, therefore, may have contained residue levels that were below the detectability of the method, either because of greater sample inter-

Table 4. Recovery of Compound 1080 from fortified samples (1 g) of bait

Added, ppm	Found, ppm	Recovery, %	Av. rec., % (std dev.)
1	0.13	13	15 (2.8)
	0.17	17	
2	0.28	14	15 (1.4)
	0.32	16	
10	2.0	20	24 (4.9)
	2.7	27	
100	39	39	39 (0.5)
	40	40	
	39	39	
	39	39	
400	112	28	31 (2.9)
	112	28	
	132	33	
	132	33	
800	264	33	43 (6.8)
	352	44	
	352	44	
	396	49	

Table 5. Recovery of fluoroacetic acid from silica gel cleanup and micro-distillation step

Added, μg	Distn only, % (std dev.)[a]	Silica gel + distn, % (std dev.)[a]	Silica gel only,[b] %
1	38 (12)	26 (3.5)	68
5	55 (3.5)	—	—
10	55 (9.5)	32 (5.4)	58
100[c]	51 (3.5)	—	—
Av.	47	29	63

[a] N = 4.
[b] Corrected for recovery from distillation.
[c] A 10% aliquot of distillate was derivatized.

Table 6. Analysis of tissues from animals collected in a field test

Sample type	No. of samples analyzed	No. of samples 1080 detected	Range, ppm
Ground squirrels			
Pretreat	4	none	—
Post-treat	20	18	1–150[a]
Other rodents[b]			
Pretreat	8	none	—
Post-treat	29	15	2–76[a]
Cottontail rabbits			
Pretreat	4	none	—
Post-treat	13	4	4–20
Small birds			
Pretreat	11	none	—
Post-treat	11	3	1–4
Doves and quail			
Pretreat	12	none	—
Post-treat	22	none	—
Predators[c]			
Pretreat	6	none	—
Post-treat	16	1	2
Bait (oat groats)			
Untreated	4	none	—
Treated	22	19	5–730

[a] High values are from analysis of cheek pouch contents (containing Compound 1080-treated grain bait) of ground squirrel and pocket mouse.

[b] Includes mostly kangaroo and wood rats, and pocket and deer mice.

[c] Includes mostly coyotes, bobcats, skunks, and raccoons.

ference or because of degradation of the compound. Some samples were from animals that were suspected or known to have died from other causes and, therefore, did not contain Compound 1080. The three treated bait samples in which residues were not found were collected from the field 6 months after application and degradation of the compound through weathering would be expected over this duration of time.

The sensitivity of the method was demonstrated in the analysis of tissues from coyotes given small acute doses of Compound 1080. Residues calculated to be near the 0.1 ppm level were found in the muscle, kidney, and heart from a coyote dosed with 0.5 mg/kg (5 mg total). The gas chromatograms obtained were similar to those shown in Fig. 3. Residues (< 0.1 ppm) were also detected in 10 g samples of tissues from coyotes dosed with 0.16 mg/kg (1.7 mg total) which is near the LD_{50} level.

Despite the limited recoveries, the method facilitates sensitive and reproducible measurements of Compound 1080 in samples considerably smaller than those required by other methods. In addition to its sensitivity, the method is less time consuming than others. The use of two GC columns allows greater specificity, and additional confirmation can be obtained by GC-MS.

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Extraction of Pentachlorophenol and Tetrachlorophenol Residues from Field-Contaminated Carrots and Potatoes: Comparison of Several Methods

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A number of solvent extraction systems were investigated for their efficiency in removing residues of pentachlorophenol (PCP) and tetrachlorophenol (TCP) from field-contaminated carrots and potatoes. Greatest numerical yields of PCP and TCP, for both carrots (9 and 5 ng/g, respectively) and potatoes (147 and 11 ng/g, respectively), were obtained by Soxhlet extraction with acidified acetone for 44 hr. Within the limits of experimental error, equivalent results could be obtained by using a 20 hr Soxhlet extraction with acidified acetone for carrots, and a 5 min blending with acidified acetone for potatoes. Extracts were reacted with diazoethane to form the ethyl ethers of PCP and TCP, followed by cleanup on a Florisil column. A gas-liquid chromatograph equipped with a ^{63}Ni linearized electron capture detector was used for identification and quantitation.

Pentachlorophenol (PCP) has been widely used as a defoliant, herbicide, insecticide, fungicide, and wood preservative. Tetrachlorophenol (TCP) is the major impurity in commercial wood preservative formulations of PCP, probably arising from incomplete chlorination of phenol during the manufacturing process. Other impurities which have been found in commercial PCP (1–4) and their possible metabolic products (5, 6) were not the subject of this investigation.

Several samples of carrots and potatoes, which had been stored for 1–2 months in bins treated 6–8 years previously with a commercial preparation of PCP, were received by our laboratory for analysis because of a peculiar taste. Analysis of the samples according to the method of Yip (7) for vegetables showed measurable levels of PCP and TCP. In order to check extraction efficiencies, replicate samples from larger macerates were extracted and analyzed by the above method and by the method described by Yip (7) for dairy products, meat, etc. Substantially larger amounts of PCP were found in the samples analyzed by Yip's method for dairy products (Table 1).

Because the method for dairy products gave a better yield of PCP and TCP than did the method for vegetables and because, by combining the samples on hand, reasonably large composite macerates of carrots and potatoes would be available, this was deemed to be an excellent opportunity to study extraction efficiencies of a number of techniques on field-contaminated samples. The results of the study are reported here.

METHOD

Apparatus

(a) *Chromatographic tubes.*—28 mm od × 650 mm, with Teflon plug and coarse fritted disk.

(b) *Centrifuge tubes.*—13 mL capacity, graduated, conical, with Teflon-lined screw caps.

(c) *Gas chromatograph.*—Tracor Model 550 equipped with pulse-modulated ^{63}Ni electron capture detector. Operating conditions: injector 220°C, column 180°C, detector 280°C, 10% methane in argon as carrier and purge gases flowing at 50 and 20 mL/min, respectively.

(d) *Column.*—1.1 m × 6 mm od borosilicate glass; injector port length packed with silanized glass wool, balance packed with 10% OV-101/QF-1 (1+4) on 80–100 mesh Gas-Chrom Q.

Reagents

(a) *Standards.*—PCP, purity not stated (Eastman Kodak Co., Rochester, NY 14650); 2,3,4,6-tetrachlorophenol (*m*-TCP), technical grade (Fluka AG, Chemische Fabrik, CH-9470 Buchs, Switzerland). Prepare individual solutions in acetone containing 10 μg each PCP and TCP/mL. Place 100 μL of each in separate 250 mL evaporator flasks and prepare their ethyl ethers as described under *Derivatization*. Purify derivatized products as described under *Cleanup*. Transfer to separate 100

Table 1. PCP and TCP (ng/g) extracted from composite samples of potatoes and carrots, using the methods of Yip for A, vegetables, and B, dairy products

Sample	Extraction method	PCP	TCP
Potatoes	A	77	9
	B	94	9
Carrots	A	6	1
	B	9	2

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mL volumetric flasks and dilute to 100 mL with hexane. The PCP standard prepared in this manner was gas chromatographically pure. The TCP standard contained 17% PCP as an impurity.

(b) *Solvents.*—Acetone, benzene, chloroform, ethyl ether, ethanol, methanol, methylene chloride, and petroleum ether: distilled in all-glass systems.

(c) *Acetonitrile and hexane.*—Nonspectro grade, distilled in glass (Burdick & Jackson, Muskegon, MI 49442).

(d) *Paraffin solution.*—1% paraffin oil in benzene.

(e) *Diazoethane solution.*—Prepare in ethyl ether from *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine according to method of Stanley (8).

(f) *Florisil.*—60–100 mesh, 5% w/w deactivation; prepare according to Currie (9).

(g) *Sodium sulfate.*—Anhydrous, coarse, granular. Heat at 600°C for 48 hr to drive off impurities. Cool and store in glass containers with Teflon-lined caps.

(h) *Water.* — Charcoal-filtered and petroleum ether-extracted as in ref. 9, or other interference-free water.

(i) *Hydrochloric acid.*—1*N* HCl in water prepared as in (h).

(j) *Sulfuric acid.*—10% in water prepared as in (h).

(k) *Sodium chloride solution.*—NaCl to saturation in water prepared as in (h).

(l) *Sodium hydroxide.*—3% w/v NaOH in water prepared as in (h).

Extraction

Method 1.—Yip's method for vegetables and potatoes was modified to include the addition of acid to release possible conjugates and we added a second extraction step.

Weigh 100 g macerated sample into blender container. Add 120 mL acetonitrile and 5 mL 1*N* HCl and blend 3 min at medium speed. Transfer blender contents to 350 mL borosilicate glass Büchner funnel fitted with medium porosity fritted disk, and collect filtrate in 500 mL Erlenmeyer flask, using aspirator vacuum as necessary. Rinse blender with 20 mL acetonitrile and pour rinse onto contents of funnel. Collect rinse and return macerate to blender container. Add 100 mL acetonitrile, blend 3 min at medium speed, filter, and wash, as described above, into same Erlenmeyer flask. Transfer combined filtrates to 1 L evaporator flask and evaporate acetonitrile on rotary evaporator at 35°C. To remaining aqueous phase, add 30 mL 3% NaOH, and transfer to 500 mL separatory funnel with two 25 mL rinses of water and one 50 mL rinse of $CHCl_3$. Add 10 mL each NaCl solution and ethanol to funnel. Stopper funnel and shake contents vigorously 1 min, venting as necessary. Let layers separate 5–10 min and discard $CHCl_3$ layer. Wash aqueous layer with additional 30 mL $CHCl_3$. Acidify aqueous layer with 30 mL 10% H_2SO_4, add 50 mL $CHCl_3$, and mix contents of funnel vigorously 1 min. Let layers separate and drain organic phase through 50 g anhydrous granular Na_2SO_4, collecting it in 250 mL evaporator flask. Extract aqueous phase 2 more times as described above, using 30 mL portions of $CHCl_3$. Rinse Na_2SO_4 twice with 25 mL portions of $CHCl_3$. Add 1 drop paraffin oil solution and evaporate just to dryness on rotary evaporator at 35°C. Proceed to derivatization step.

Method 2.—The method described by Yip for dairy products and meat was modified to include a second extraction step.

Weigh 100 g macerated sample into blender container. Add 200 mL $CHCl_3$, 100 mL water, 10 mL 10% H_2SO_4, and 20 g NaCl. Blend 3 min at medium speed. Transfer contents of blender container to 500 mL centrifuge bottle and centrifuge 15 min at 1600 rpm. Siphon off lower $CHCl_3$ layer into 1 L separatory funnel. Return remaining contents of centrifuge bottle to blender container with three 50 mL rinses of centrifuge bottle with $CHCl_3$. Blend, centrifuge, and siphon organic layer as described above. Add 100 mL water, 35 mL 3% NaOH, 20 mL ethanol, and 10 mL NaCl solution to separatory funnel. Proceed from "Stopper funnel . . ." in Method 1.

Method 3.—The method for extraction of chlorophenoxyacetic acid herbicides described in ref. 10 was modified to include a second extraction step.

Weigh 100 g macerated sample into blender container and add 150 mL ethyl ether, 50 mL petroleum ether, 25 mL ethanol, and 10 mL 10% H_2SO_4. Blend, centrifuge, and siphon organic layer into 1 L separatory funnel as described in Method 2. Re-extract macerate with 100 mL ethyl ether plus 40 mL petroleum ether as described in Method 2. To combined extracts in separatory funnel, add 10 g NaCl, 10 mL ethanol, 35 mL 3% NaOH, and 100 mL water. Mix contents of separatory funnel vigorously and let layers separate 5–10 min. Drain aqueous layer into second 1 L separatory funnel containing 25 mL ethyl ether. Vigorously mix contents of second funnel and let layers separate 5–10 min. Discard organic layer in first funnel and collect aqueous layer from second funnel in it. Discard second organic layer. Proceed from "Acidify aqueous layer . . ." in Method 1.

Method 4.—The acetone blending procedure of Luke *et al.* (11) for multi-residue screening was modified to include a second extraction step.

Weigh 100 g macerated sample into blender container add 200 mL acetone and 5 mL 10% H_2SO_4. Blend 2 min at medium speed. Filter as in Method 1 using 20 mL acetone rinse of blender

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container. Return solids to blender container, add 120 mL acetone, and 2 mL 10% H_2SO_4, and blend 2 min at medium speed. Filter sample and rinse blender container as above. Proceed from "Transfer combined filtrates . . ." in Method 1.

Method 5.—Acidify 25 g macerated sample with 2 mL 1*N* HCl and place in extraction thimble. Place thimble in Soxhlet extractor fitted with 500 mL boiling flask containing 300 mL petroleum ether and a few boiling chips. Adjust reflux rate to obtain 3–4 cycles/hr. Extract for 20 hr, let solvent cool, and filter through cotton wool plug fitted in powder funnel (wash funnel, plug, and flask with two 30 mL portions of $CHCl_3$ before use) into 500 mL evaporator flask. Rinse funnel with 25 mL petroleum ether. Proceed from "Add 1 drop paraffin oil solution . . ." in Method 1.

Method 6.—Acidify 25 g macerated sample with 2 mL 1*N* HCl and place in extraction thimble. Place thimble in Soxhlet extractor fitted with 500 mL boiling flask containing 300 mL methanol and a few boiling chips. Adjust reflux rate to obtain 3–4 cycles/hr. Extract for 20 hr, let solvent cool, and transfer to 1 L evaporator flask with two 25 mL rinses of methanol. Evaporate methanol on rotary evaporator at 35°C. Proceed from "To the remaining aqueous phase . . ." in Method 1.

Method 7.—Proceed as in Method 6, substituting acetone for methanol.

Method 8.—Proceed as in Method 4 but extend blending time to 5 min.

Method 9.—Proceed as in Method 6, substituting $CHCl_3$–methanol (9+1) for methanol.

Method 10.—Proceed as in Method 7 but extend extraction time to 44 hr.

Derivatization

Dissolve evaporator flask residue in 10 mL ethyl ether. Add 2 mL diazoethane solution and let reaction proceed 30 min at room temperature. Evaporate just to dryness on rotary evaporator at 30°C. Dissolve in 25 mL petroleum ether before column chromatography.

Cleanup

Using powder funnel, place 50 g Florisil into chromatographic tube. Tap tube with wooden rod or heavy cardboard tube to settle adsorbent. Place 20 g Na_2SO_4 on top of Florisil layer and tap tube to settle layers. Pour sample onto dry column, using funnel to facilitate transfer. When petroleum ether meniscus reaches top of Na_2SO_4 layer, rinse beaker, funnel, and sides of column with small portions of petroleum ether totaling 25 mL. Place 500 mL evaporator flask under tube, again let solvent meniscus reach top of Na_2SO_4 layer, and elute column with 150 mL 25% CH_2Cl_2–petroleum ether at flow rate of 5 mL/min. Add 1 drop paraffin oil solution and evaporate just to dryness on rotary evaporator at 35°C. Quantitatively transfer residue containing ethyl ethers of PCP and TCP to graduated centrifuge tube, using small rinses of petroleum ether. Reduce volume to ca 0.1 mL with gentle stream of filtered air, adjust to 5 mL with hexane, cap tube, and save for gas-liquid chromatography (GLC).

Detection and Quantitation

Inject 5 μL (equivalent to 24 or 100 mg) sample extract onto GLC column. Quantitatively measure by comparing peak heights to those obtained by injecting same volume of standards. If response from sample exceeds full scale deflection of strip-chart recorder, dilute sample extract so that response is on scale. Detector response is linear over at least 1 full-scale width of recorder.

Under GLC conditions described earlier, 50 pg PCP and TCP (as ethyl ethers) injected onto column gave detector responses of ca 40% full scale (10 cm). Based on response of 4% of full-scale, injection of ca 25 mg extracted sample results in a lower detection limit of 0.2 ng/g for PCP and TCP. Retention times were 3.2 and 1.6 min, respectively, for PCP and TCP.

Results and Discussion

Extraction methods which required filtration to separate the extractant from the solid material were modified by the addition of a second extraction step in order to circumvent the need for applying volume correction factors to the data. In most cases, the original published methods include the application of such a factor. The choice of extractant for the second step was, in each case, partially arbitrary, but the choice did represent an attempt to approximate the conditions of the first extraction step. Because materials had already been extracted from the sample in the first step, it was felt that exact duplication of the initial extraction conditions would be virtually impossible.

The amounts of PCP and TCP extracted from potato composite Sample 1 by Methods 1–7 are given in Table 2. No carrot samples were analyzed in this series because carrot supplies were more limited.

A cursory examination of the data reveals that the methods suggested by Yip involving other types of substrates are relatively ineffective for field-contaminated potatoes, as are ethyl ether–petroleum ether blending and Soxhlet extraction with petroleum ether. It is possible that the low yield obtained with Yip's acetonitrile extraction system is due to evaporation with acetonitrile from the extract. In the present study, PCP and TCP losses were noted on the

Table 2. PCP and TCP (ng/g) extracted from composites of field-contaminated potato samples by Methods 1–7[a]

Method	PCP	TCP
1	50	6
2	60	7
3	62	7
4	73	8
5	62	7
6	69	8
7	85	11

[a] See text for description of methods. Blending time was 3 min for Methods 1, 2, and 3, and 2 min for Method 4. Soxhlet extraction time was 20 hr in all cases.

evaporation of acetonitrile from water–acetonitrile mixtures fortified with PCP and TCP. However, it was not possible to minimize these losses in extracts by first forming the sodium salts of the phenols because the addition of base to the extract caused uncontrollable foaming at the evaporation stage. Acetone was the most effective solvent used. As might be expected, Soxhlet extraction with acetone was superior to blending with acetone. However, even acetone blending more efficiently extracted PCP than did Soxhlet extraction with methanol, which ranked third in efficiency.

The effect of contact time of the acetone extraction solvent with the sample was investigated with a second composite sample of potatoes and with a composite sample of carrots. Because the amount of sample available was sufficient, the chloroform–methanol (9+1) solvent system described by Bowman and Beroza (12) for the extraction of a phenolic metabolite of leptophos was modified slightly as described in Method 9, and investigated.

As shown by a comparison of Methods 4 and 7–10 in Table 3, increased contact time between sample and solvent effectively increased PCP yields, but not TCP, from the potato macerate.

The greatest effect was noted with the blending technique. Comparatively speaking, Soxhlet extraction with chloroform–methanol was no more effective than the Soxhlet extraction with methanol used in the first series of tests.

The results of the same series of tests conducted on the composited carrot sample are also shown in Table 3. In contrast to the potato samples, blending with acetone was the least efficient extraction system investigated, and extension of the blending time doubled the TCP extraction but did not affect PCP extraction. Soxhlet extractions in carrots with chloroform–

Table 3. PCP and TCP (ng/g) extracted from composites of field-contaminated potato and carrot samples by Methods 4 and 7–10

Method	PCP	TCP
Potatoes		
4	109	9
7	140	11
8	128	9
9	98	10
10	147	11
Carrots		
4	3	1
7	7	5
8	3	2
9	7	5
10	9	5

methanol or acetone were equally efficient when both were carried out for 20 hr, whereas chloroform–methanol was considerably less efficient for potatoes. As for potatoes, when Soxhlet extraction with acetone was extended to 44 hr, more PCP was recovered from the sample but the extracted amount of TCP was unaffected.

Thus the method of choice for both carrots and potatoes is a 44 hr Soxhlet extraction with acidified acetone. Where total elapsed time for analysis is a critical factor, PCP and TCP can be extracted from potatoes by blending samples for 5 min with acidified acetone with losses of only 13% PCP and 18% TCP. The only alternative for carrots is to shorten the Soxhlet extraction time to 20 hr with corresponding comparative losses of 22% PCP and 0% TCP.

Although fortified samples are useful for assessing the general validity of an analytical procedure, the value of assessing extraction efficiencies when field-incurred residues are encountered is illustrated for PCP in carrots in Table 4. The 5 min acidified acetone-blending technique, which extracted only 33% of the amount of PCP extracted by 44 hr Soxhlet extraction, allowed recovery of essentially 100% (after correction for naturally occurring levels) of spiked PCP.

Table 4. Recoveries of PCP from spiked carrot samples using Method 8

Fortification level, ng/g	Blank, ng/g	PCP found, ng/g	Recovery, %
10	3.3	14.1	108
100	3.3	101	98

Table 5. Recoveries of PCP from spiked potatoes by Method 8 and from spiked carrots by Method 7

Fortification level, ng/g	Blank, ng/g	PCP found, ng/g	Recovery, %
	Potatoes		
1	0.2	1.0	80
1	0.2	1.1	90
10	0.2	9.8	96
10	0.2	9.7	95
100	0.2	93.0	93
100	0.2	99.0	99
	Carrots		
20	4.0	22.4	92
20	0.7	22.4	108
200	4.0	180	88
200	0.7	214	107

The general validity of the 5 min acidified acetone blending extraction procedure for potatoes and of the 20 hr Soxhlet extraction with acidified acetone for carrots was verified for PCP by using samples fortified with several levels of PCP. After correction for naturally occurring levels, recoveries ranged from 70 to 99% for potatoes (mean 92%) and from 89 to 108% for carrots (mean 99%). Individual data are presented in Table 5. From these data it may be speculated that the differences in extraction efficiency between Methods 8 and 10 for potatoes and between Methods 7 or 9 and Method 10 for carrots are due to variations in recovery. A brief investigation of the etherification reaction revealed a deviation of ±12% from the mean for PCP and TCP (6 determinations), which is sufficient to account for the variability noted in the fortification studies.

The GLC conditions used in the tests did not allow us to distinguish the *m*- and *p*-isomers of TCP. *P*-values (13), between acetonitrile and hexane (1+1), were not sufficiently different (0.62 and 0.67 for *m*- and *p*-TCP, respectively) to allow distinguishing the 2 isomers on this basis. A second GLC column packed with a 2 + 3 mixture of OV-101/QF-1 on 80–100 mesh Gas-Chrom Q also did not resolve the *m*- and *p*-isomers, although retention times relative to aldrin were altered in comparison to the 1 + 4 column described under *Apparatus* (Table 6). However, based on the general reactivity of phenol, one would normally expect the TCP isomers to be present in the order of *m*- > *o*- > *p*-, at least in commercial preparations of PCP, and for this reason, *m*-TCP was used for quantitation of TCP. Technical PCP, obtained from a commercial wood preservative preparation, contained 93.1% PCP, 6.7% *m*- (+*p*-?) TCP, and 0.1% *o*-TCP. The general term TCP was preferred because of the inability to distinguish *m*- and *p*-TCP and in recognition of the possibility that the degradation rate of the 2 might be quite different. A very small peak with the same retention time as *o*-TCP was noted in approximately 10% of the samples, but the peak in each case was so small as to preclude accurate quantitation or an accurate determination of *P*-value.

Table 6. *P*-values (acetonitrile–hexane 1+1) and relative retention data (vs. aldrin) for the ethyl ethers of PCP and 2,3,4,5-TCP (*o*-TCP), 2,3,4,6-TCP (*m*-TCP) and 2,3,5,6-TCP (*p*-TCP)

Ethyl ether	*P*-value	Relative retention time OV-101/QF-1 (1+4)	Relative retention time OV-101/QF-1 (2+3)
PCP	0.75	0.50	0.56
o-TCP	0.49	0.40	0.44
m-TCP	0.62	0.25	0.28
p-TCP	0.67	0.25	0.28

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COLOR ADDITIVES

High Performance Liquid Chromatographic Determination of Sulfanilic Acid, Schaeffer's Salt, 4,4'-(Diazoamino)-Dibenzenensulfonic Acid, and 6,6'-Oxybis(2-Naphthalenesulfonic Acid) in FD&C Yellow No. 6: Collaborative Study

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Eight laboratories participated in a collaborative study of a high performance liquid chromatographic procedure for determining sulfanilic acid, Schaeffer's salt, 4,4'-(diazoamino)-dibenzenesulfonic acid (DAADBSA), and 6,6'-oxybis(2-naphthalenesulfonic acid) (DONS) in FD&C Yellow No. 6. The standard deviations for one analysis in any one laboratory for sulfanilic acid at 0.2%, Schaeffer's salt at 0.26%, DAADBSA at 0.1%, and DONS at 1.0% were 0.015%, 0.014%, 0.018%, and 0.077%, respectively. The method has been adopted as official first action.

The Certification Branch of the Division of Color Technology, Food and Drug Administration, determines the uncombined intermediates sulfanilic acid (SA) and Schaeffer's salt (SS) and 2 reaction by-products, 4,4'-(diazoamino)-dibenzenesulfonic acid (DAADBSA) and 6,6'-oxybis(2-naphthalenesulfonic acid) (DONS) in batches of FD&C Yellow No. 6 by high performance liquid chromatography (HPLC). The HPLC method was first reported for the determination of SA, SS, and DONS by Singh (1). Bailey and Cox (2) reported that Singh's method could also be used to determine DAADBSA. Marmion (3) modified the HPLC method to determine DAADBSA exclusively and conducted a collaborative study for DAADBSA.

The HPLC method as used by the Certification Branch is different from these published methods. This currently used method was subjected to a collaborative study. The results of the study are presented in this report.

This report of the Associate Referee was presented at the 93rd Annual Meeting of the AOAC, Oct. 15–18, 1979, at Washington, D.C.

Sulfanilic Acid (SA), Schaeffer's Salt (SS), 4,4'-(Diazoamino)-dibenzenesulfonic Acid (DAADBSA), and 6,6'-Oxybis(2-Naphthalene Sulfonic Acid) (DONS) in FD&C Yellow No. 6

34.A01 Apparatus

(a) *Liquid chromatograph.*—With gradient elution capability and automatic sampler, e.g., DuPont 830 with Model 838 programmable gradient and Model 834 automatic sampler with 5 μL sampling loop. Operating conditions: eluant flow rate, 0.6 mL/min; temp., ambient; gradient profile, program IX 70, Fig. 1, is scribed on program charts provided with Model 838 programmable gradient and run at time setting of 40 min; equilibration time, 10 min between each gradient run.

(b) *Detectors.*—(1) DuPont 254 nm detector (low pressure Hg source) with attenuation set at 0.08 AUFS. (2) DuPont 385 multiwave detector (medium pressure Hg source and 325–385 nm filter) with attenuation set at 0.04 AUFS.

(c) *Column.*—DuPont Zipax SAX column (Cat. No. 852950005). Condition new column by heating 50 hr at 50°C with 0.01M $Na_2B_4O_7$ flowing at 1000 psi and then letting it rest unused 2 weeks.

(d) *Spectrophotometer.*—For use in visible and UV range.

34.A02 Reagents

(a) *Eluants.*—(1) *Primary eluant.*—0.01M $Na_2B_4O_7$. (2) *Secondary eluant.*—0.2M $NaClO_4$ in 0.01M $Na_2B_4O_7$.

(b) *Std solns.*—(1) *Sulfanilic acid (SA).*—Approx. 0.25 mg/mL H_2O. (2) *Schaeffer's salt (SS).*—Approx. 0.35 mg Na salt of 2-naphthol-6-sulfonic acid/mL H_2O. (3) *6,6'-Oxybis(2-naphthalene sulfonic acid) (DONS).*—Approx. 1.2 mg/mL H_2O. (4) *4,4'-(Diazoamino)-dibenzenesulfonic acid (DAADBSA).*—Approx. 0.15 mg/mL 0.2% NaOH.

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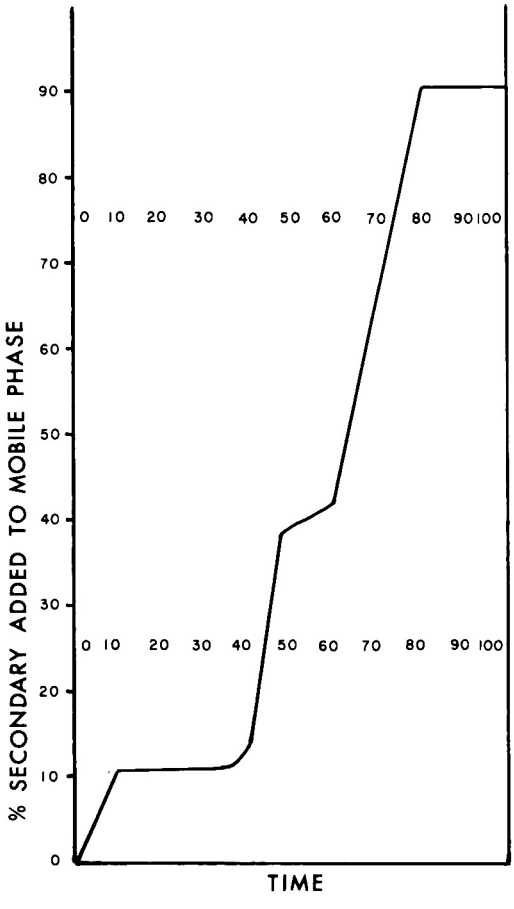

FIG. 1—Gradient profile program for separating intermediates and reaction by-products from FD&C Yellow No. 6 by HPLC.

Det. exact concn of std solns (*1*)–(*3*) from UV spectra of dild aliquots in H_2O; that of std soln (*4*) from visible spectra of dild aliquots in 1% NaOH. Approx. *a* (mg/mL, 1 cm) at specified nm are: (*1*) 81.6, 247; (*2*) 292, 231; (*3*) 179, 238; and (*4*) 86.5, 410.

34.A03 Suitability Test

Prep. test soln contg 1% FD&C Yellow No. 6 and, relative to the FD&C Yellow No. 6, 0.2% SA, 0.3% SS, 0.1% DAADBSA, and 0.5% DONS. Set parameters as in **34.A01(a)** and run blank gradient (no injection) and test soln. Compare chromatogram to that of Fig. 2. If similar resolution is not attained, adjust operating parameters to those needed to resolve compds.

34.A04 Calibration

For each calibrating soln, dissolve 0.500 g FD&C Yellow No. 6 (free of SA, SS, DAADBSA, and DONS) in ca 25 mL H_2O, add 5 mL 0.01*M* $Na_2B_4O_7$ and aliquots indicated in Table 1, and dil. to 50 mL with H_2O.

Calc. concns of compds being detd as wt % of FD&C Yellow No. 6. Calc. % concns in calibration solns as:

$$C = V \times C' \times 0.2$$

where V = vol. std soln taken; C' = concn std soln, **34.A02(b)**, detd spectrophtrc. in mg/mL; and 0.2 = 100 (%)/500 (mg).

Measure areas of peaks from SA, SS, and DONS, using 254 nm detector, and of DAADBSA, using 360 nm detector. Areas may be obtained from integrator or by multiplying peak ht by peak width at half peak ht. Plot % concn of each compd against peak areas. Use calibration solns 1–5 to det. retention times of the 4 compds.

34.A05 Determination

Weigh 0.500 g sample, dissolve in ca 25 mL H_2O, add 5 mL 0.1*M* $Na_2B_4O_7$, and dil. to 50 mL with H_2O. Chromatograph sample solns interspersed with calibration solns. Compare chromatograms of samples with those of calibration solns. Identify by retention times and measure areas of peaks corresponding to the 4 compds. Use calibration plots to det. % SA, SS, DAADBSA, and DONS.

Collaborative Study

Eight analysts from 8 laboratories participated in the study. Each analyst was sent the method to be followed; small amounts of SA, SS, DAADBSA, and DONS to use in calibrating; a calibrating sample which contained no significant amounts of the compounds being determined; 8 test samples; a practice sample; spectrophotometric curves of SA, SS, DAADBSA, and DONS; and a labeled chromatogram of the practice sample, Fig. 2.

The sample lot used for the calibrating sample was also used for the preparation of the test Samples 2–7. These test samples were prepared by adding known amounts of the compounds being determined in solution to a known amount of the FD&C Yellow No. 6 in solution. The instruments and operating parameters used are given in Table 2. The amounts of added compounds are listed in Table 3. The dye was brought out of solution by drying in a vacuum oven at 70°C and about 23 in. Hg. The dried samples were ground and mixed. Test Samples 1 and 8 are samples of the 2 lots currently undergoing pharmacological testing.

The collaborators were asked to follow the

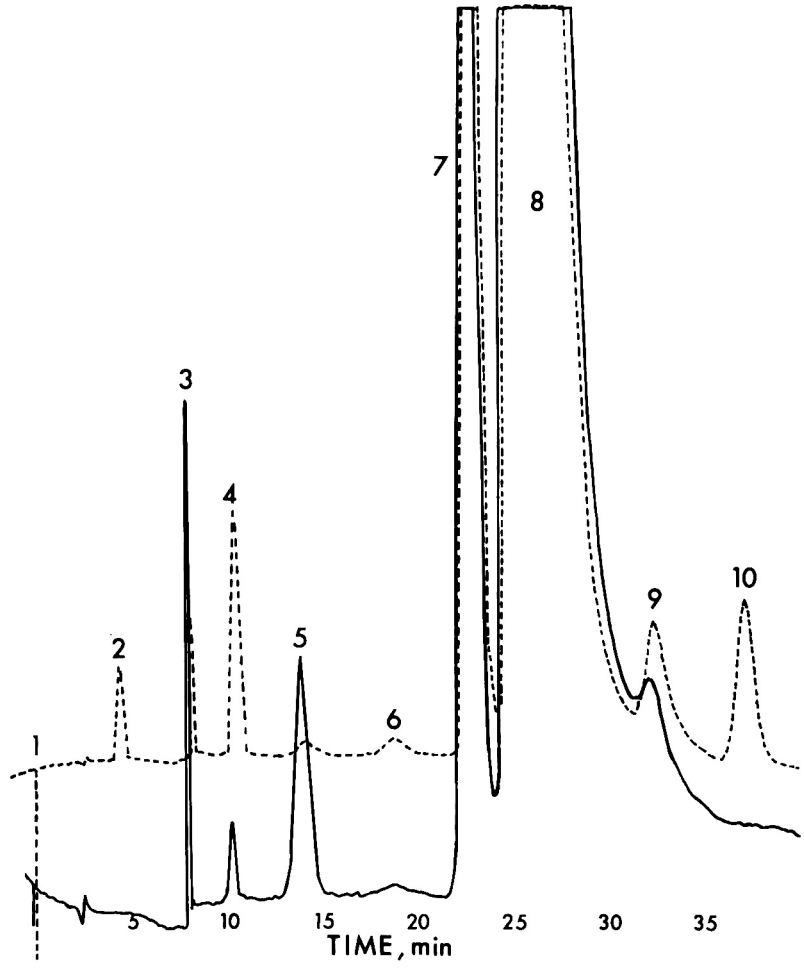

FIG. 2—Chromatogram of FD&C Yellow No. 6 (peak 8).

1 = injection; 2 = sulfanilic acid; 3 = gradient front peak; 4 = Schaeffer's salt; 5 = DAADBSA; 6 = unknown; 7 = higher sulfonated subsidiary; 9 = unknown; 10 = DONS. Top tracing from 254 nm detector; bottom tracing from 325–385 nm detector.

Table 1. Preparation of calibration solutions

	Standard solution, mL			
Soln	SA	SS	DAADBSA	DONS
1				
2	5			
3		5		
4			5	
5				5
6	1	2	3	4
7	2	3	4	1
8	3	4	1	2
9	4	1	2	3

method as closely as possible. Samples 1 and 2 were to be analyzed in duplicate; the other samples were to be analyzed only once. Two report sheets were provided: one for reporting the amounts found in test samples and the average retention times from the calibration solution, the other for reporting the instrument, column, and operating parameters used. The SA, SS, and DONS results were to be reported to the nearest 0.01% and the DAADBSA results to the nearest 0.001%. The collaborators were asked to submit a chromatogram of the practice sample.

Table 2. Operating conditions used by collaborators

Coll.	Instrument	Volume injected, μL	Flow rate, mL/min	Gradient profile	Equilibration time, min	Detectors nm	Detectors AUFS	Method of estimating peaks
1	Waters Assoc. Model 440	5	1.0	8 (slow start) 0–90%, 60 min	20	254 365	0.1 0.1	[a]
2	DuPont Model 830	5	0.6	0–75% at 1%/min Mode 2 (slow start)	10	254 360	0.02 0.02	[a]
3	DuPont Model 830	10	0.6	0–100% at 2%/min Mode 3 (slow start)	15	254 365	0.04 0.1	[a]
4	DuPont Model 850	10	0.6	linear 0–11% (4 min) linear 11–38% (2.5 min) linear 38–42% (4.0 min) linear 42–95% (6.0 min)	15	254 365	0.01 0.01	integrator
5	DuPont Model 830, 834 automatic sampler, 838 programmable gradient	10	1.0	Exp. x^2 concave 0–100%; 40 min	10	254	0.08	[a]
6	DuPont Model 830, 834 automatic sampler, 838 programmable gradient	20	0.6	IX 70 See Fig. 2	10	254 325	0.08 0.04	[a]
7	DuPont Model 830	5	0.6	0–90% Mode 2 (slow start)	10	254 365	0.08 0.08	[a]
8	DuPont Model 830, 834 automatic sampler, 838 programmable gradient	5	0.6	IX 70 See Fig. 2	10	254 325–385	0.08 0.04	[a]

[a] Area of peaks estimated by multiplying peak height times peak width at ½ peak height.

Results and Comments

All collaborators used a Zipax SAX column. The eluants specified in the method were used by all collaborators except Collaborator 2 who used a secondary eluant of 0.5*M* $NaClO_4$ in 0.01*M* $Na_2B_4O_7$. The collaborators prepared the calibration solutions and sample solutions as specified except Collaborator 6, who prepared the solutions at a concentration of 0.5 g in 200 mL of 0.01*M* $Na_2B_4O_7$ instead of 50 mL because the injection volume was 4 times greater. As shown in Table 1, the collaborators made other changes in the operating conditions. These changes were necessary because of the different instruments used.

The collaborators made the following comments:

Collaborator 1 had no difficulties.

Collaborator 2 commented that the study was performed over 5 days. The stock solution was analyzed before and after the 5-day period. No decomposition was seen.

Collaborator 3 had mechanical problems with the automatic sampler, and calibration solutions were analyzed 18–24 hr after the aliquots were added.

Collaborator 4 adapted the segment style gradient of the DuPont 850 instrument to reproduce the gradient profile of Fig. 2. He found a 15-min equilibration period to work best with this instrument. He used a Rheodyne Universal Septumless injector and rinsed it with distilled water between samples.

Collaborator 5 did not have a multiwavelength detector and therefore determined DAADBSA with the 254 nm detector and reported results to 2 decimal places. He had some difficulty constructing the baseline for the DONS peak. This baseline was taken as the tangent drawn from the start of the DONS peak to the tail of the small peak eluting after the DONS peak.

Collaborator 6 saw a peak eluting after DONS in all test and calibration solutions but not in any blank gradients.

Collaborator 7 experienced difficulties in conditioning columns; he tried conditioning 2 columns, neither of which performed adequately. For the study he used an unconditioned column which had been used for about 6 months. Because this column did not resolve SA, all values for SA were omitted. The SA was not seen in

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Table 3. Collaborative results for per cent sulfanilic acid, Schaeffer's salt, DAADBSA, and DONS in FD&C Yellow No. 6

Sample	Added, %	Collaborator 1	2	3	4	5	6	7	8
Sulfanilic Acid									
1a	—[a]	0.03	0.02	0.03	0.02	0.02	0.03	—[c]	0.024
1b	—[a]	0.03	0.02	—[c]	0.02	0.02	0.03	—[c]	0.026
2a	0.20	0.21	0.18	0.23	0.18	0.20	0.18	—[c]	0.20
2b	0.20	0.20	0.18	—[c]	0.19	0.21	0.18	—[c]	0.20
3	0.20	0.20	0.20	0.20	0.18	0.20	0.17	—[c]	0.18
4	0.10	0.08	0.11	0.11	0.09	0.10	0.10	—[c]	0.10
5	0.10	0.09	0.10	0.11	0.09	0.09	0.10	—[c]	0.092
6	—[b]	<0.01	0.00	—[c]	<0.005	0.01	<0.01	—[c]	0
7	0.20	0.16	0.16	0.20	0.17	0.18	0.16	—[c]	0.17
8	—[a]	0.03	0.02	0.03	0.02	0.02	0.03	—[c]	0.025
Schaeffer's Salt									
1a	—[a]	0.03	0.03	0.02	0.03	0.03	0.02	<0.1	0.032
1b	—[a]	0.03	0.02	—[c]	0.03	0.03	0.02	<0.1	0.028
2a	0.30	0.26	0.27	0.25	0.27	0.26	0.25	0.25	0.29
2b	0.30	0.29	0.27	—[c]	0.25	0.27	0.26	0.27	0.28
3	0.30	0.26	0.26	0.25	0.25	0.25	0.24	0.40	0.27
4	0.15	0.12	0.13	0.11	0.13	0.13	0.15	0.18	0.15
5	0.15	0.14	0.13	0.14	0.13	0.12	0.11	0.16	0.14
6	—[d]	<0.01	0.01	—[c]	<0.02	0.01	<0.01	<0.1	0.00
7	0.30	0.27	0.28	0.32	0.27	0.27	0.29	0.40	0.30
8	—[a]	0.04	0.03	0.04	0.04	0.03	0.04	<0.1	0.037
DAADBSA									
1a	—[a]	0.04	0.017	0.032	0.031	<0.04	0.023	<0.05	0.033
1b	—[a]	0.04	0.022	—[c]	0.032	0.06	0.020	<0.05	0.032
2a	0.103	0.11	0.105	0.104	0.101	0.10	0.143	0.09	0.101
2b	0.103	0.10	0.088	—[c]	0.103	0.08	0.40	0.09	0.103
3	0.101	0.11	0.074	0.096	0.099	0.08	0.127	0.14	0.098
4	0.054	0.05	0.052	0.051	0.054	0.06	0.050	0.08	0.054
5	0.054	0.05	0.068	0.062	0.053	0.06	0.045	0.085	0.052
6	0.096	0.10	0.086	0.107	0.096	0.11	0.128	0.13	0.098
7	—[e]	<0.01	0.00	—[c]	trace	<0.04	0.000	<0.05	0.000
8	—[a]	<0.01	0.00	0.005	trace	<0.04	0.004	<0.05	0.001
DONS									
1a	—[a]	0.30	0.16	0.23	0.28	0.20	0.25	0.30	0.29
1b	—[a]	0.30	0.14	—[c]	0.24	0.22	0.21	0.33	0.27
2a	1.02	1.00	0.93	1.03	0.92	1.10	1.00	0.84	1.01
2b	1.02	0.95	0.84	—[c]	0.93	1.18	0.86	0.84	0.99
3	1.00	0.93	0.94	0.98	0.96	1.03	0.95	1.08	0.97
4	0.53	0.42	0.34	0.50	0.50	0.50	0.44	0.40	0.54
5	0.53	0.49	0.42	0.52	0.47	0.47	0.45	0.46	0.52
6	1.00	0.96	0.80	1.19	0.99	1.13	0.94	1.12	1.01
7	—[f]	0.02	0.00	—[c]	<0.03	<0.02	<0.01	<0.10	0.00
8	—[a]	0.05	0.00	—[c]	<0.03	0.02	0.04	<0.10	0.00

[a] Samples No. 1 and 8 are the current pharmacology samples AA 3003 and AA 8634, respectively.
[b] No sulfanilic acid added to this sample.
[c] No results reported.
[d] No Schaeffer's salt added to this sample.
[e] No DAADBSA added to this sample.
[f] No DONS added to this sample.

either calibration solutions or test samples. The 365 nm detector was used at 0.08 AUFS because of high noise level at 0.04 AUFS. He used syringe injection and expressed the opinion that non-repetitive injection and particulate matter associated with system injection may have influenced his results. He also resolved other peaks in the practice sample not present in the

chromatogram provided. These peaks were not present in all samples and varied in area when present. This collaborator's chromatogram of the practice sample showed that SS and DAADBSA peaks were much broader than these peaks in any other collaborator's chromatograms and that the baseline was unstable. Because of these problems and the erratic variations in his results, his results were not included in the statistical evaluation.

Collaborator 8 used a Hewlett-Packard 97 calculator with a program for linear regression curve fitting, instead of plotting the calibration data.

Table 3 presents the results found in the test samples for SA, SS, DAADBSA, and DONS, respectively. Table 4 presents the average retention times from the calibration solution as found by the collaborators. The retention times of these compounds varied. This variation was most likely caused by the different instruments and different operating conditions used. Two collaborators, 6 and 8, used the same gradient profile and flow rate. The retention times found by these 2 collaborators are in good agreement. The difficulty of variation in retention time was foreseen in writing the method. The calibration procedure requires that each compound be chromatographed alone to identify the retention time of that compound.

Samples 1 and 8, currently being tested pharmacologically, were included in the study to obtain a good estimate of the amount of these intermediates and side reaction products present in these pharmacology samples. Collaborators were asked to analyze Sample 1 in duplicate to provide the between-laboratory and within-laboratory standard deviations at the low level present in this sample. Samples 2 and 3 were prepared at the proposed specification limit. Together these samples provide the between-laboratory and within-laboratory standard deviations at the proposed specification limit. Samples 4 and 5 were prepared at one-half the proposed specification limit and they provide the between-laboratory and within-laboratory standard deviations at one-half the proposed specification limit. Sample 6 was prepared without SA and SS and at the specification limit for DAADBSA and DONS. Comparison of the results for Sample 6 to those for Samples 2 and 3 provides a test of the effect of the presence of SA on DAADBSA and SS on DONS. Sample 7 was prepared without DAADBSA or DONS and at specification limit for SA and SS. Comparison of the results for Sample 7 to those for Samples 2 and 3 provides a test of the effect of the presence of DAADBSA on SA and DONS on SS. SA and DAADBSA are related compounds because SA is a decomposition product of DAADBSA. SS and DONS are related compounds because DONS is formed from 2 molecules of SS.

Table 4. Average retention times from calibration solutions found by collaborators

Coll.	Retention time (min)			
	SA	SS	DAADBSA	DONS
1	7	28	34	58
2	4.2	15.2	24.1	65.4
3	4.8	12.9	20.2	50.7
4	3.07	13.1	16.22	39.02
5	2.5	10.8	15.7	34.0
6	3.6	8.6	12.7	36.4
7	N.R.	11.5	17.0	50.0
8	3.6	10.0	12.4	37.5

Table 5 presents the statistical analysis of the collaborative results for SA. The between-laboratory standard deviation shown is calculated from the between-laboratory component of variability only. The between-laboratory variability contributes significantly to the overall variability for Samples 2a + 2b + 3 and 1a + 1b. In Samples 4 + 5, the within-laboratory variability accounts for most of the overall variability.

Table 6 presents the statistical analysis of the collaborators' results for SS. The between-laboratory variability contributes significantly to the overall variability for Samples 1a + 1b. In Samples 2a + 2b + 3 and 4 + 5, the within-laboratory variability accounts for most of the overall variability.

Table 7 presents the statistical analysis of the collaborative results for DAADBSA. The between-laboratory variability contributes significantly to the overall variability for Samples 2a + 2b + 3 and 1a + 1b. In Samples 4 + 5, the within-laboratory variability accounts for most of the overall variability.

Table 8 presents the statistical analysis of the collaborative results for DONS. The between-laboratory variability contributes significantly to the overall variability for Samples 2a + 2b + 3 and Samples 4 + 5 and Samples 1a + 1b.

For the 7 laboratories used in the statistical analysis, there is no indication that the known

Table 5. Statistical analysis of collaborative results for sulfanilic acid

	% SA				
	Standard deviation				
Samples	Between-lab.	Within-lab.	One lab. 1 observation	Av. found	Av. recd., %
1a + 1b	0.0048	0.00058	0.0048	0.025	—[a]
2a + 2b + 3	0.0111	0.0098	0.015	0.194	97
4 + 5	0.0072	0.0067	0.0098	0.098	98
6	—	—	0.0048	0.006	—[b]
7	—	—	0.015	0.171	85
8	—	—	0.005	0.025	—[a]

[a] Samples 1 and 8 are the current pharmacology Samples AA3003 and AA8634, respectively.
[b] No sulfanilic acid added to this sample.

Table 6. Statistical analysis of collaborative results for Schaeffer's salt

	% SS				
	Standard deviation				
Samples	Between-lab.	Within-lab.	One lab. 1 observation	Av. found	Av. recd., %
1a + 1b	0.0038	0.0031	0.0049	0.026	—[a]
2a + 2b + 3	0.0086	0.011	0.014	0.262	87
4 + 5	0.0	0.015	0.015	0.131	87
6	—	—	0.0063	0.01	—[b]
7	—	—	0.019	0.29	97
8	—	—	0.0047	0.037	—[a]

[a] Samples 1 and 8 are the current pharmacology Samples AA3003 and AA8634, respectively.
[b] No Schaeffer's salt added to this sample.

Table 7. Statistical analysis of collaborative results for DAADBSA

	% DAADBSA				
	Standard deviation				
Samples	Between-lab.	Within-lab.	One lab. 1 observation	Av. found	Av. recd., %
1a + 1b	0.0095	0.0060	0.011	0.032	—[a]
2a + 2b + 3	0.016	0.0088	0.018	0.104	101
4 + 5	0.0029	0.0054	0.0061	0.054	100
6	—	—	0.013	0.104	108
7	—	—	0.021	0.016	—[b]
8	—	—	0.014	0.01	—[a]

[a] Samples 1 and 8 are the current pharmacology samples.
[b] No DAADBSA added to this sample.

duplicates (2a + 2b) are closer than the coded duplicates (2 and 3).

The average amount of SA found in Sample 7 is significantly less than the average amount of SA found in Samples 2 and 3. However, the amount of SA found in Samples 2 and 3 did not exceed the amount added nor was the amount of DAADBSA added in Samples 2 and 3 less than the amounts added as might be expected if the DAADBSA had decomposed to SA. The low recovery of SA in Sample 7 is most likely due to loss during sample preparation.

The average amount of SS found in Sample 7 is significantly more than the average amount found in Samples 2 and 3. This result is most likely explained by loss of SS from Samples 2 and 3 during sample preparation.

The average amounts of DAADBSA and DONS found in Sample 6 were not significantly different from the average amounts of DAADBSA and DONS in Samples 2 and 3.

On Samples 1 and 8, the results given as "less than" a given amount were used in the statistical analysis as the given amount, which

Table 8. Statistical analysis of collaborative results for DONS

Samples	% DONS				
	Standard deviation				
	Between-lab.	Within-lab.	One lab. 1 observation	Av. found	Av. recd., %
1a + 1b	0.048	0.019	0.052	0.24	—[a]
2a + 2b + 3	0.059	0.050	0.077	0.98	96
4 + 5	0.044	0.032	0.054	0.47	89
6	—	—	0.128	1.003	100
7	—	—	0.012	0.013	—[b]
8	—	—	0.021	0.023	—[a]

[a] Samples 1 and 8 are the current pharmacology samples.
[b] No DONS added to this sample.

Table 9. 95% Confidence limits on an individual analysis

Sample	SA	SS	DAADBSA	DONS
1a + 1b	0.025±0.012	0.023±0.013	0.032±0.028	0.24 ±0.13
2a + 2b + 3	0.194±0.039	0.262±0.036	0.104±0.046	0.98 ±0.20
4 + 5	0.098±0.025	0.131±0.039	0.054±0.016	0.47 ±0.14
6	0.006±0.012	0.01 ±0.016	0.104±0.033	1.00 ±0.33
7	0.171±0.039	0.29 ±0.049	0.016±0.054	0.013±0.031
8	0.025±0.013	0.037±0.012	0.01 ±0.036	0.023±0.054

is in most cases the limit of detection for that laboratory. Therefore, the standard deviations shown are an indication of the variability of the limits of detection among laboratories.

The 95% confidence limits on an individual analysis are presented in Table 9. The true value of the per cent component for one analysis will be enclosed by these limits with 95% confidence. This also means there is a 5% chance that these limits do not contain the true value. The confidence limits demonstrate a wider range than had been anticipated for this method.

This variability should be considered when results of analysis by this method indicate that a batch of FD&C Yellow No. 6 does not conform to the specifications for the substances involved in this study.

It is recommended that the method for SA, SS, DAADBSA, and DONS be adopted as official first action.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee G and was adopted by the Association. Their reports will appear in *J. Assoc. Off. Anal. Chem* (1980) **63**, March issue.

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J. King, Crompton & Knowles Corp., Gilbraltar, PA

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N. Richfield-Fratz, Food and Drug Administration, Washington, DC

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FISH AND OTHER MARINE PRODUCTS

Fish Species Identification By Thin Layer Polyacrylamide Gel Isoelectric Focusing: Collaborative Study

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A method for fish species identification by isoelectric focusing of sarcoplasmic proteins on LKB Ampholine PAGplates, pH 3.5–9.5, was collaboratively studied to determine whether photographs of protein patterns from authentic species could be used to identify unknown samples. Seven collaborators were sent 8 unknown samples in duplicate to identify from an 8 × 10 in. photograph of an Ampholine PAGplate showing standard protein patterns from 14 species of fish. The 7 collaborators identified the unknown samples with 93% accuracy. Eight of 14 monkfish samples were not identified correctly and showed protein patterns different from the protein patterns shown in the photographic standard. All of the incorrectly identified samples showed the same protein pattern. The other 7 unknown species were all correctly identified. The method has been adopted as official first action.

Conventional zone electrophoresis methods for fish species identification are limited in their effectiveness as routine species identification tests. Variations in stabilizing media composition, sample application technique, separation time, applied voltage or current, and analyst's skill can cause day-to-day variations in the protein patterns. Current AOAC methods for fish species identification based on starch gel (1), disc gel (2), and cellulose acetate electrophoresis (3) require that authentic samples be analyzed along with unknown samples to obtain a valid identification. A previous attempt to use photographic standards with the cellulose acetate electrophoresis method was unsuccessful; only 39% of the unknowns were correctly identified (4).

Thin layer isoelectric focusing (TLIEF), because it is an equilibrium separation technique, has been shown to be an excellent technique for the differentiation of fish species (5). A collaborative study was conducted to determine whether photographs of TLIEF protein patterns from authentic species could be used to reliably identify unknown samples.

Seven collaborators were each sent 8 unknown samples in duplicate. The unknown samples consisted of 16 frozen fish fillets, each coded with a separate letter. Collaborators were also supplied with three pH 3.5–9.5 LKB Ampholine PAGplates and an 8 × 10 in. photograph of an Ampholine PAGplate showing protein patterns from 14 authenticated fish species. This "library gel" was prepared according to the proposed method. The collaborators were instructed to identify all unknown samples by comparing the protein patterns they obtained with the patterns shown on the photograph.

Thin Layer Polyacrylamide Gel Isoelectric Focusing Method—Official First Action

(*CAUTION:* Polyacrylamide gels may contain small amts acrylamide monomer which is harmful if absorbed thru skin. Wear disposable vinyl gloves when handling gels. See also *Caution* in **18.077**.)

18.A01 Apparatus

(**a**) *Thin layer isoelectric focusing.*—LKB 2117 Multiphor for Electrofocusing (LKB Instruments, Inc., 12221 Parklawn Dr, Rockville, MD 20852) or equiv. isoelectric focusing app. with similar sized cooling platform and electrode geometry.

(**b**) *Power supply.*—Const-power type capable of maintaining const power of ≥1–30 watts. Const-voltage type may be used provided voltage is manually increased at intervals to maintain const power level.

(**c**) *Constant temperature circulator.*—Capable of circulating H_2O or antifreeze soln thru cooling platform at 0–10°.

(**d**) *Trays.*—Plastic or metal covered. Min. size 125 × 260 × 20 mm, to hold fixing, staining, destaining, and preserving solns.

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(e) *Glass plates.*—125 × 260 mm, to support gels during drying.

(f) *LKB Ampholine PAGplates.*—pH 3.5–9.5 (LKB Instruments, Inc.).

18.A02 Reagents

(a) *Fixing soln.*—Mix 150 mL MeOH and 350 mL H_2O. Add 17.25 g sulfosalicylic acid and 57.50 g trichloroacetic acid. Discard after one use.

(b) *Destaining soln.*—Mix 500 mL alcohol and 160 mL HOAc. Dil. to 2 L with H_2O.

(c) *Staining soln.*—Dissolve 0.230 g Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, 2200 Wright Ave, Richmond, CA 94804) in 200 mL reagent (b). Heat to 50–60° in covered container in H_2O bath before use. Discard after one use.

(d) *Preserving soln.*—Add 50 mL glycerol to 500 mL reagent (b).

(e) *Anode soln.*—1*M* H_3PO_4.

(f) *Cathode soln.*—1M NaOH.

(g) *Insulating fluid.*—Light paraffin oil (Saybolt viscosity 125/135).

18.A03 Preparation of Sample

Ext sarcoplasmic proteins as in **18.088**, first par., or as in **18.095**. Centrf. all exts, 1500 × g for 15 min, at 4° or filter thru Whatman No. 1 paper in refrigerator.

18.A04 Determination

Step 1.—Place template supplied with Ampholine PAGplates on cooling platform over thin layer reagent (g). Avoid air bubbles.

Step 2.—Open PAGplate package by cutting around edge. Do not cut or crush gel. PAGplate may be cut in ½ or ⅓, if less than whole gel is required, and remainder may be resealed in package for later use. Leave clear plastic film covering PAGplate.

Step 3.—Lift PAGplate by ends of protruding plastic support. Place PAGplate on cooling platform, over template, using addnl oil, (g). Keep gel surface clean. Remove clear plastic film covering gel surface.

Step 4.—Place electrode strip (supplied with PAGplates) on clean glass plate. Sat. strip with 1*M* H_3PO_4, (e). Strip should show wet surface. Place wet strip on PAGplate surface at anode position marked on template. Cut protruding ends of strip with clean sharp scissors.

Step 5.—Sat. another electrode strip as in step 4, but with 1*M* NaOH, (f). Place wet strip on PAGplate surface at cathode position marked on template. Cut protruding ends of strip with sharp clean scissors.

Step 6.—Pick up sample application piece with clean forceps. Apply 5 μL sample ext to piece, using micropipet. Place piece on PAGplate surface within sample application area marked on template at cathode. Sixteen samples may be applied if long side (10 mm) of piece is placed parallel to cathode strip. Twenty-four samples may be applied if short side (5 mm) of piece is parallel to cathode strip. Leave min. of 5 mm between adjacent samples.

Step 7.—Place electrode lid (for electrofocusing across width of gel) on gel with Pt wires centered on electrode strips. Connect leads to electrofocusing app. terminals. Observe proper polarity.

Step 8.—Place safety cover in place and connect leads to power supply. Observe proper polarity.

Step 9.—Set power supply conditions.—(*1*) *For const-power power supply.*—Power, 30 watts; max. voltage, 1.5 kV; max. current, 50 mA; for 1.5 hr. Then 1 watt, 1.5 kV; 50 mA for 16 hr.

Reduce power level to 10–15 watts for 2 hr if cooling platform temp. is 10–25°, or if less than whole PAGplate is used. When using other power supply conditions, use max. voltage, current, and time to ensure all proteins have reached equilibrium.

(*2*) *Constant-voltage power supply* — Set to const-voltage mode, initial setting 300 V, and increase voltage by 100 V at 30 min intervals. Experimentally det. max. voltage and experiment time for each set of conditions.

Using either const-power or const-voltage supplies, min. experiment time is time required for all proteins to reach their isoelectric points. Apply 10 μL Ferritin, Horse Spleen (10–15 μg/μL) on sample application strips placed at anode and cathode sample application areas as indicator of equilibrium focusing. When anode and cathode zones merge and focus, equilibrium conditions are attained.

Step 10.—Switch off power supply after 30 min; remove safety cover and electrode lid. Remove sample application pieces. Replace safety cover and lid and continue sepn.

Step 11.—Switch off power supply at end of sepn and remove safety cover and electrode lid. Remove electrode strips from gel surface gently with forceps. If strip sticks to gel, cut thru gel (not plastic support) at inner edges of electrode strips. Discard strips.

Step 12.—Place PAGplate immediately in fixing soln, (a), 30 min.

Step 13.—Rinse PAGplate in destaining soln, (b), 5 min.

Step 14.—Stain PAGplate 10 min in hot (60°) staining soln, (c).

Step 15.—Destain PAGplate with several changes of reagent (b). Gel background should be clear after destaining overnight. Gently remove any pptd dye from PAGplate surface with cotton wool soaked in reagent (b).

This report of the Associate Referee was presented at the 93rd Annual Meeting of the AOAC, Oct. 15–18, 1979, at Washington, DC.

Table 1. Summary of collaborative study results for thin layer isoelectric focusing method of fish species identification using photographic standards

Species	Sample Code	Collaborator[a] 1	2	3	4	5	6	7	No. correct/ No. samples
Ocean perch	D	C	C	C	C	C	C	C	14/14
	L	C	C	C	C	C	C	C	
Monkfish	S	I	I	C	C	C	I	I	6/14
	X	I	I	C	C	I	C	I	
Pollack	V	C	C	C	C	C	C	C	14/14
	Q	C	C	C	C	C	C	C	
Cod	A	C	C	C	C	C	C	C	14/14
	I	C	C	C	C	C	C	C	
Wolffish	T	C	C	C	C	C	C	C	14/14
	Y	C	C	C	C	C	C	C	
Cusk	U	C	C	C	C	C	C	C	14/14
	Z	C	C	C	C	C	C	C	
Haddock	B	C	C	C	C	C	C	C	14/14
	J	C	C	C	C	C	C	C	
Whiting	C	C	C	C	C	C	C	C	14/14
	K	C	C	C	C	C	C	C	
No. Correct/No. samples		14/16	14/16	16/16	16/16	15/16	15/16	14/16	104/112

[a] C = correct identification; I = wrong identification.

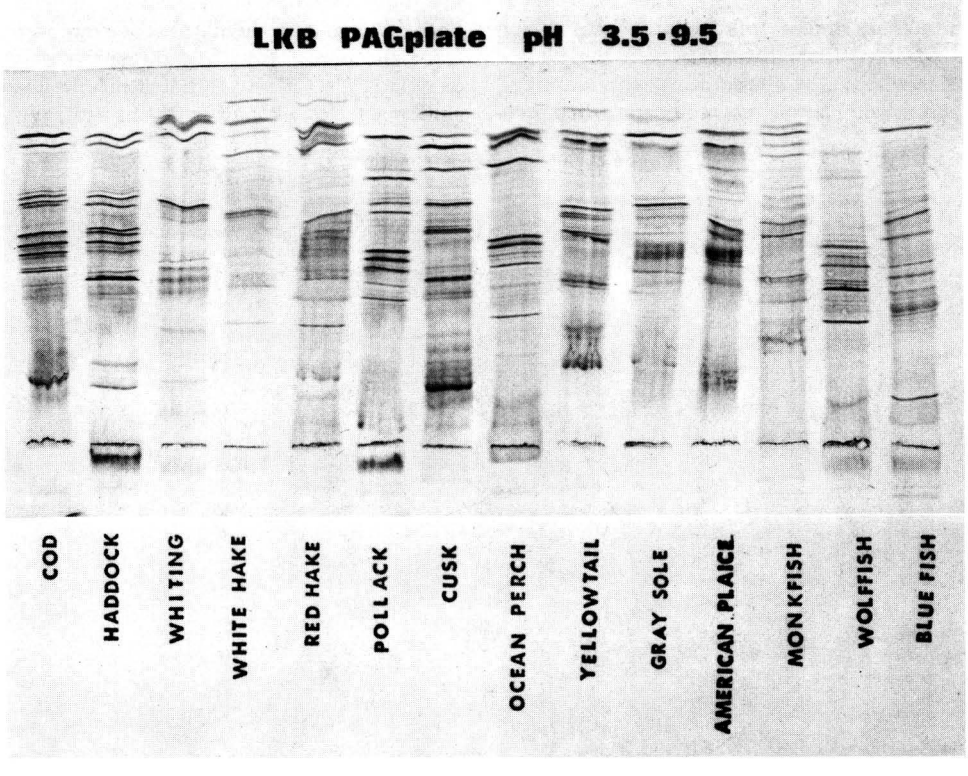

FIG. 1—Sarcoplasmic protein patterns from 14 species of fish focused on a pH 3.5–9.5 LKB Ampholine PAGplate.

From left to right: cod (*Gadus morhua*), haddock (*Mellanogrammus aegelfinus*), whiting (*Merluccius bilinearis*), white hake (*Urophysis tenuis*), red hake (*Urophysis chuss*), pollock (*Pollachius virens*), cusk (*Brosme brosme*), ocean perch (*Sebastes marinus*), yellowtail (*Limanda ferruginea*), gray sole (*Glyptocephalus cynoglossus*), American plaice (*Hippoglossoides platessoides*), monkfish (*Lophius americanus*), wolffish (*Anarhichas lupus*), and bluefish (*Pomatomus saltatrix*).

Step 16.—Compare protein patterns on destained PAGplate with patterns on previously prepd PAGplate with authentic species. Patterns may also be compared after step 19.

Step 17.—Place destained PAGplate in preserving soln, (**d**), 60 min.

Step 18.—Dry PAGplate on glass plate at room temp. overnight. Protect gel surface from dust, etc.

Step 19.—Roll sheet of plastic (supplied with PAGplates) onto dried PAGplate, avoiding air bubbles. Store dried PAGplate in dark. Dried PAGplate may be stapled into notebook.

Results and Discussion

Table 1 summarizes the results obtained from 7 collaborators. Two collaborators reported 16 correct identifications. Two collaborators reported 15 correct, and 3 collaborators reported 14 correct of 16 samples each. The overall average for correct identification was 93%. All wrong identifications were monkfish samples. Collaborators who identified the monkfish samples incorrectly returned gels showing patterns for monkfish similar to each other but which were different from the pattern in the photograph (see Fig. 1). Collaborators who identified the monkfish sample correctly returned gels showing patterns for monkfish that matched the pattern in the photograph.

Figure 2 shows the PAGplate returned by one collaborator who incorrectly identified only one monkfish sample. It can be seen that the correctly identified monkfish sample matches the monkfish pattern in Figure 1, while the other monkfish sample had a different pattern that did not match any pattern on the standard photograph. The other 7 species were all identified correctly. Since 2 distinct patterns were obtained for the monkfish samples, it is thought that 2 species of monkfish were inadvertently obtained when the collaborative study samples were collected. All of the fish species collected, except the monkfish, were obtained as whole fish and thus were easily authenticated. Monkfish is normally sold as monktail because only the tail portion of the fish is retained. The standard reference book for *Fishes of the Gulf of Maine* (6) lists only one species of monkfish (*Lophius americanus*) as native to this area. Perhaps, some of the monktails were imported from another area with a different native species. It is also possible that 2 morphologically similar species are native to this area and have

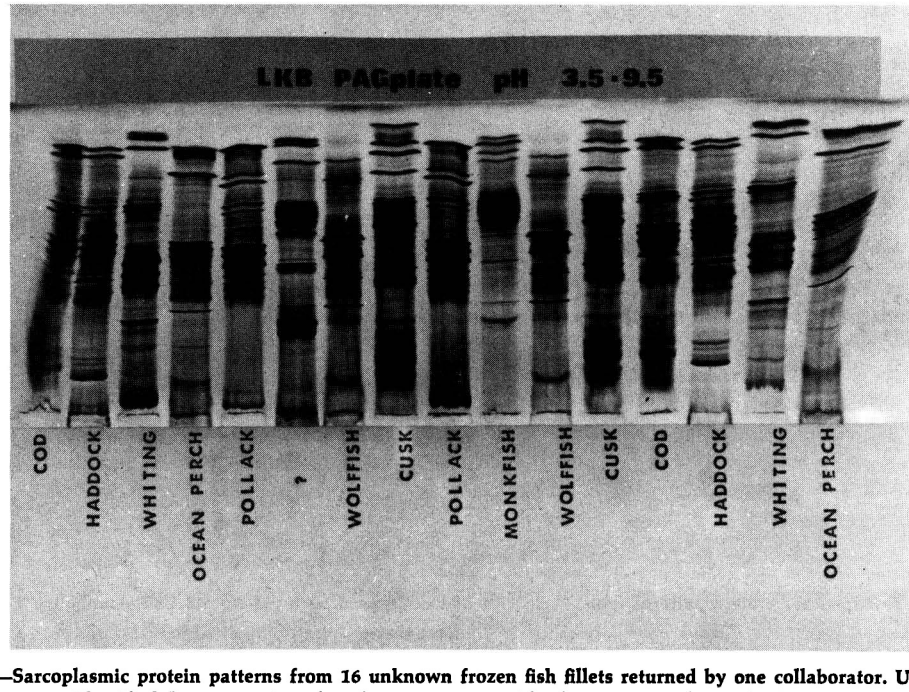

FIG. 2—Sarcoplasmic protein patterns from 16 unknown frozen fish fillets returned by one collaborator. Unknown samples were identified by comparing the above patterns with the patterns shown in Fig. 1. Note that the unidentified sample does not match the monkfish pattern in Fig. 1.

not previously been recognized as separate species. Disregarding the results for the monkfish samples, because of the problem in authentication, gives 100% correct identification for the remaining 7 species. Additional work is underway to determine the exact nature of the different monkfish patterns. Until such time as this is resolved, monkfish can be identified by matching either of the 2 patterns.

Based on the results of this collaborative study, it is recommended that the proposed method be adopted as official first action.

Acknowledgments

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The recommendation of the Associate Referee was approved by the Referee and Subcommittee C and was adopted by the Association. Their reports will appear in *J. Assoc. Off. Anal. Chem.* (1980) **63**, March issue.

Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service.

FOOD ADDITIVES

Confirmation of Low μg/kg Amounts of Volatile *N*-Nitrosamines in Foods by Low Resolution Mass Spectrometry

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Volatile *N*-nitrosamines were confirmed in foods at concentrations of less than 10 μg/kg by full scan low resolution mass spectrometry. Ground samples were vacuum-distilled from mineral oil and condensed in liquid nitrogen-cooled vapor traps. The thawed distillate was extracted, the extract was cleaned up and concentrated, and the *N*-nitrosamine content was determined by combined gas chromatography–thermal energy analysis. Positive samples were further cleaned up, trapped from a gas chromatographic column, and purged into a gas chromatograph-mass spectrometer for qualitative confirmation by full scan low resolution mass spectrometry. This procedure was applied to foods spiked at 1 μg/kg and to fried commercial bacons with volatile *N*-nitrosamine contents of 2 to 5 μg/kg.

Several reports have recently been published which indicate that carcinogenic *N*-nitrosamines (NAs) occur in some foods (1, 2). The regulatory, economic, and possible public health implications of these findings mandate that positive reports be confirmed by the most reliable methods available. The subcommittee of the International Agency for Research on Cancer (IARC) dealing with *N*-nitrosamine analysis has concluded that combined gas chromatography-mass spectrometry (GC-MS) is the most satisfactory technique available (3).

Both high and low resolution GC-MS have been successfully applied to confirmation of NAs in foods (4). Some authors have questioned the adequacy of low resolution GC-MS for confirmation of trace amounts of *N*-nitrosamines in complex samples and consider high resolution GC-MS as the only reliable method for confirmation (5). The *N*-nitrosamine analysis subcommittee of the IARC has not recommended one method over the other.

The thermal energy analyzer (TEA) is a highly selective detector for *N*-nitroso compounds (6). However, false positive findings have been reported and hence there is need for qualitative confirmation of positive responses (7, 8). The quantitative accuracy of the TEA instrument has, however, been demonstrated in world-wide studies to be as good as mass spectrometry (9, 10).

The purpose of this paper is to detail an analytical scheme in which volatile NAs are first quantitated by combined GC-TEA and subsequently qualitatively confirmed by full spectrum low resolution GC-MS. The method was demonstrated in foods spiked with volatile NAs at 1 μg/kg. In addition, we present quantitative data and qualitative confirmation of volatile NAs in fried commercial bacon at levels of less than 10 μg/kg.

METHODS

Apparatus

(Note: Glassware catalog numbers refer to VWR Scientific Inc. (1978) unless otherwise noted.)

(a) *Distillation apparatus.*—See Fig. 1. (*1*) 1 L round bottom flask, 20/40 tapered joint, with thermometer well (29129-326); (*2*) stopcock, high vacuum (59277-049); (*3*) large vacuum vapor trap with 45/50 separable joint, 41 mm × 250 mm, inner tube cut 100 mm below joint (555096-166); (*4*) small vacuum vapor trap with 29/42 separable joint, 28 mm × 200 mm (555096-100); (*5*) 28 mm od U-tube trap; (*6*) Dewar type vacuum trap (Ace Glass Co., 8757); (*7*) ball and socket joints, 28/15 (33063-163, 33063-560); (*8*) vacuum flasks, Dewar, 70 mm id × 340 mm.

(b) *Evaporative concentrator.*—Kuderna-Danish, 125 mL with 24/40 upper and 14/20 lower joints, 5 mL tube, 3-ball distilling column (Kontes Glassware, K-570000); micro-Snyder column (Kontes Glassware, K-570050).

(c) *Chromatographic column.*—14.5 mm id × 250 mm with reservoir (Kontes Glassware, K-420480-222).

(d) *GC-TEA.*—Varian Model 1400 GC interfaced to TEA™ analyzer, Model 502L, by 1.59 mm od glass-lined stainless steel tubing. GC conditions: 9 m × 3.18 mm od stainless steel column packed with 11% Carbowax 20M on 80–120 mesh Chromosorb W (AW) and fitted with 20 cm ×

Technical paper 5259, Oregon Agricultural Experiment Station, Oregon State University.

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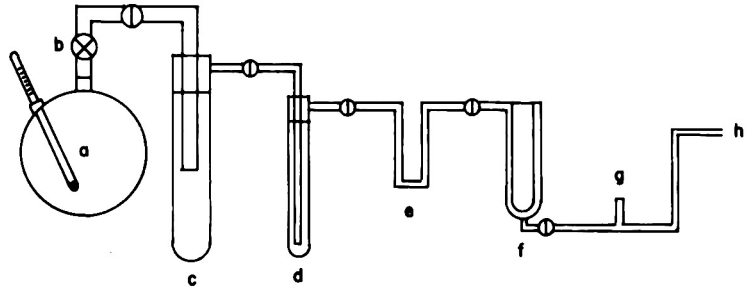

FIG. 1—Vacuum distillation apparatus: a, round-bottom flask; b, vacuum stopcock; c, large vapor trap; d, small vapor trap; e, U-tube trap; f, safety trap; g, to vacuum gauge; h, to vacuum pump.

3.18 mm od precolumn containing the same packing material and extended into injection port for on-column injection; injection port 160°C; column 100 or 160°C isothermal depending on retention time of peak; helium carrier gas 25 mL/min. TEA conditions: furnace 400°C; vacuum 4 mm Hg; isopentane-liquid N_2 slurry trap (—160°C). Mini Volume Valve (Carle Instruments, Inc. No. 5511) directs column effluent to either TEA or trapping system external to GC oven (Fig. 2).

(e) *GC-MS.*—Varian Model 1400 GC interfaced to Finnigan Model 1015C quadrupole mass spectrometer by all-glass jet separator, temperature 150°C. GC conditions: column, 180 m × 0.5 mm id Carbowax 20M glass support-coated open tubular (SCOT) column; column 120°C for NDMA and 150°C for NPyr; helium carrier gas 8 mL/min. MS parameters: emission current 450 μA; electron energy 70 eV; analyzer pressure 10^{-6} mm Hg; mass range m/z 10–100; scan rate approximately 3 sec/scan with minimal delay for increased chromatographic resolution. System Industries System 250 data system, collect data in Integration as a Function of Signal Strength (IFSS) mode. GC inlet is fitted with valving system to allow trapped compounds to be purged onto column (Fig. 2); temperature of transfer line 170°C.

Reagents

(a) *Solvents.* — (1) Dichloromethane (DCM), glass-distilled (Burdick & Jackson Laboratories, Muskegon, MI). Concentrate 100 mL of each lot to 1 mL and analyze for interfering peaks. (2) Hexane, nanograde (Mallinckrodt, St. Louis, MO).

(b) *Alumina.* — Prepare activity II alumina (Merck and Co., Rahway, NJ).

(c) *Standards.* — *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPyr) (Aldrich Chemical Co., Milwaukee, WI); *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPip) (Eastman Organic Chemicals, Rochester, NY); *N*-nitrosomorpholine (NMor) (Fluka AG, Switzerland). Prepare standards gravimetrically in hexane. (Caution: Many *N*-nitroso compounds are animal carcinogens.)

(d) *Mineral oil.*—Pharmaceutical oil containing tocopherols (Squibb, Princeton, NJ).

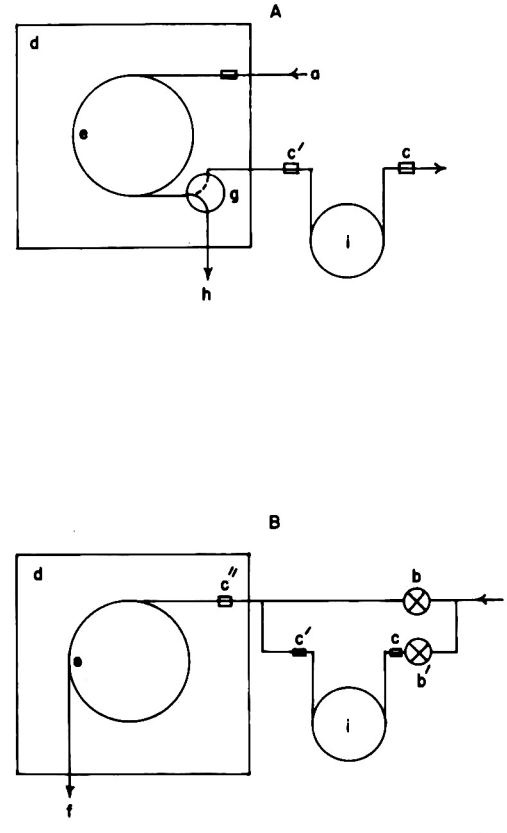

FIG. 2—Flow diagram: A, trapping *N*-nitrosamines from GC-TEA; B, purging trapped *N*-nitrosamines into GC-MS. a, gas in; b, toggle valves; c, unions; d, GC oven; e, GC column; f, to MS; g, Carle valve; h, to TEA; i, nickel trap.

(e) *Water.*—Distill in glass and do not purify through ion exchange resin.

Procedure

(a) *Sample preparation.*—Prepare bacon without nitrite by soaking pork bellies in solution of NaCl and sucrose (3:1) and smoke to internal temperature of 63°C. Store frozen, and slice into 2 mm thick slices before frying. Fry all bacon slices 3 min per side at 170°C. Fry fresh ground pork 4 min per side after forming into ca 0.5 cm thick patties. Cheese is analyzed as received and fish (Pacific hake) is analyzed raw.

Prepare all samples by freezing and grinding in liquid nitrogen, using commercial blender (Waring Model 32 BL39).

(b) *Distillation and cleanup.*—A modification of the procedure of Fine *et al.* (11) is used to isolate volatile NAs. Weigh 250 g shattered sample into distillation flask. Add 5 g ammonium sulfamate dissolved in sufficient 1*N* H_2SO_4 to lower pH of sample to <2.5 (approximately 35 mL). Add 100 mL mineral oil, and connect to distillation apparatus. Close stopcock, pump traps to vacuum >100 μm, and cool traps c, d, e (Fig. 1) with liquid nitrogen and trap f with Dry Ice-acetone. Slowly open stopcock, and pump system until vacuum again reaches 100 μm. Heat flask with electric heating mantle until contents reach 100°C and the vacuum is >100 μm (45–90 min, depending on water content). Remove heating mantle and continue vacuum for 15 min. Close stopcock and break vacuum between traps e and f.

Thaw traps c and d, and transfer distillate to 500 mL separatory funnel. Rinse each trap with three 25 mL portions of water followed by 25 mL DCM, and combine all rinses with distillates. Saturate aqueous phase with sodium sulfate, separate DCM, and further extract aqueous phase with three 25 mL portions of DCM. Wash combined DCM extracts with 25 mL each of sodium bisulfite-water (1 + 4, w/v), 3*N* HCl, 1.5*N* NaOH. Backwash sodium bisulfite and HCl washes with 10 mL DCM. Dry combined DCM over ca 10 g anhydrous Na_2SO_4 and add 1 mL hexane. Filter DCM through glass wool, wash Na_2SO_4 with 25 mL DCM, and reduce combined DCM to ca 3 mL in Kuderna-Danish evaporative concentrator in 55–60°C water bath. Fit concentrator tube with micro-Synder column and reduce contents to 1 mL under nitrogen at ambient temperature.

Inject 4–8 μL concentrate into GC-TEA immediately after injecting known amounts of standard NA solution. Repeat standard and unknown injections. Record retention times, and calculate concentration of apparent NAs on the basis of the corresponding known NA.

Further clean up those samples for GC-MS which contain GC-TEA positive peaks on 11 g slurry-packed (hexane) activity II alumina column. Quantitatively transfer concentrate from concentrator tube to column, using small rinsings of hexane. Wash column with 100 mL 10% DCM in hexane, and elute NAs with 100 mL DCM. Concentrate DCM as described above except evaporate under nitrogen to ca 250 μL.

(c) *Peak trapping and injection.*—Trap GC-TEA positive peaks onto 1.59 mm od nickel tubing as follows: Refit GC-TEA System as detailed in Fig. 2A. Bend 25.4 cm length of 1.59 mm nickel tubing into 2.5 cm diameter loop and fit ends with 1.59 mm Swagelok fittings. Connect trap (i) by union (c′) to 1.59 mm tube through oven wall, and connect to internal Carle valve (g). Cool trap in Dry Ice-methoxy ethanol bath. Inject 25–50 μL aliquots of concentrated DCM from column cleanup into GC-TEA. As recorder begins to respond to TEA-positive compound, rotate valve (g) to direct flow to cooled nickel trap (i) outside oven. After ca 2 min, rotate valve back to TEA. Repeat procedure for all 250 μL, if necessary. Disconnect trap, seal ends with caps, and store traps in Dry Ice.

Purge trapped compounds into GC-MS by refitting GC oven as detailed in Fig. 2B. With valve b′ closed and valve b open, connect nickel trap between unions c and c′ while keeping trap cold in crushed Dry Ice. Close valve b and open valve b′ while heating trap with forced air heat gun. Compare both retention times and spectra of unknowns to standard NAs run immediately before.

Results and Discussion

NAs can occur as artifacts of the analytical procedure if precautions are not taken. For example, when 50 mg dimethylamine and pyrrolidine/kg were added to non-nitrite cured bacon and 120 mg sodium nitrite/kg was added to the distillation flask, 1000 μg/kg of the corresponding NAs was formed. If 5 g ammonium sulfamate was added to the bacon, the artifacts were reduced to 1–100 μg/kg. When the pH of the bacon was reduced to less than 2.5, the artifacts were reduced to less than 1 μg/kg. Hence, both addition of ammonium sulfamate and reduction of pH as described in the procedure section are necessary to ensure against artifactural nitrosamine formation.

Injection of pure solvents into our system gave no evidence of ghosting for volatile nitrosamines. To avoid the possibility of ghosting, we suggest avoiding cold spots and introducing minimal amounts of standards.

The per cent recovery for 7 volatile NAs ranged from a low of 74 for NDMA to a high of 98 for NDPA (Table 1). The average recovery was 90%. These values represent the effi-

Table 1. Per cent recovery from bacon spiked with 10 μg/kg of each volatile *N*-nitrosamine[a]

N-Nitrosamine	$\bar{x}$	*s*
NDMA	74	7.02
NDEA	94	4.16
NDPA	98	3.29
NDBA	85	10.39
NPip	96	4.55
NPyr	92	6.36
NMor	88	6.12

[a] n = 5.

ciency of the distillation and cleanup procedures and are comparable to values reported for similar distillation procedures (12).

Raw fish, cheese, fried ground pork, and non-nitrite cured fried bacon were spiked on several occasions with NDMA and NPyr to demonstrate a lower limit of confirmation. Representative spectra of NDMA and NPyr from these foods spiked at 1 μg/kg are given in Figs 3 and 4. Also included are spectra of standards run on our instrument. The base peak in most

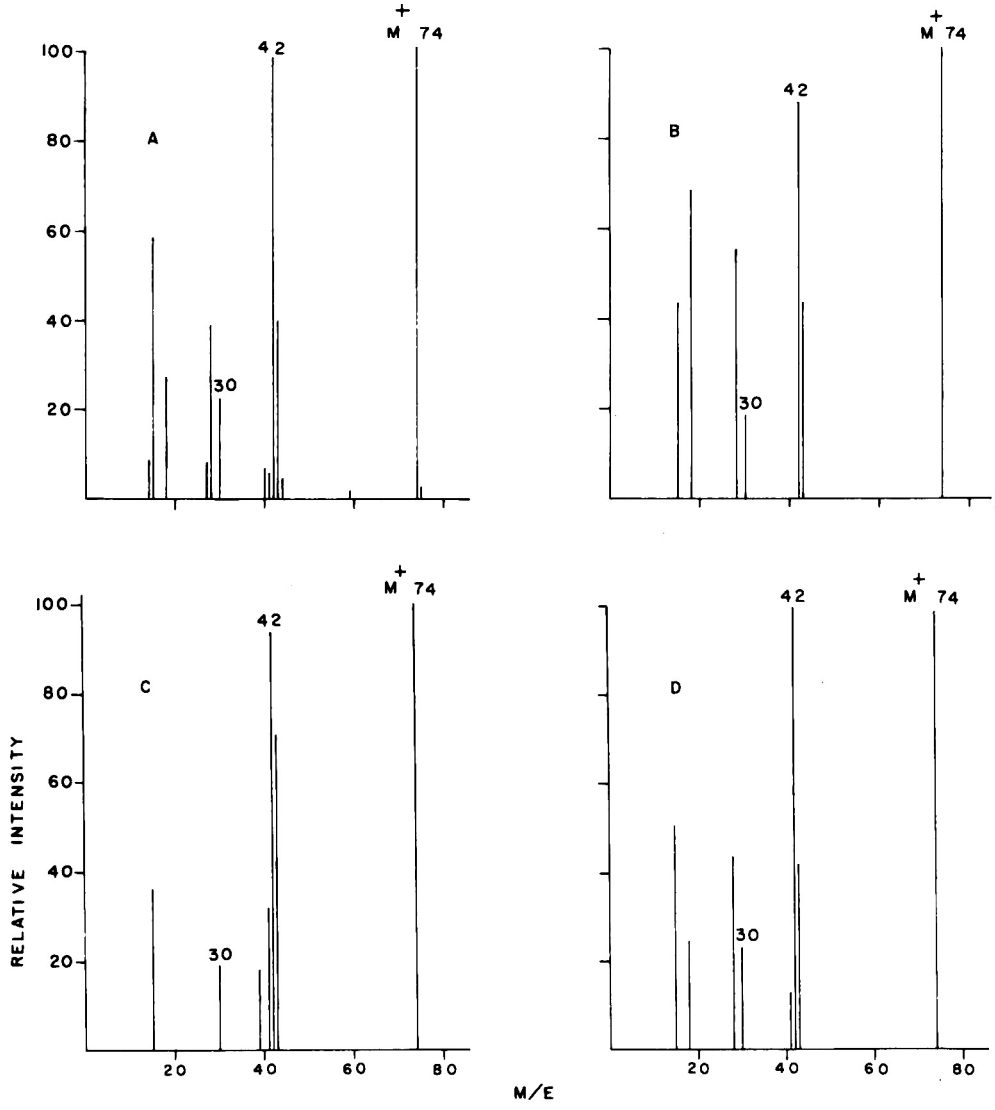

FIG. 3—Mass spectra of NDMA: A, standard; B, 1 μg/kg spiked fish; C, 1 μg/kg spiked fried pork; D, 3 μg/kg fried commercial bacon.

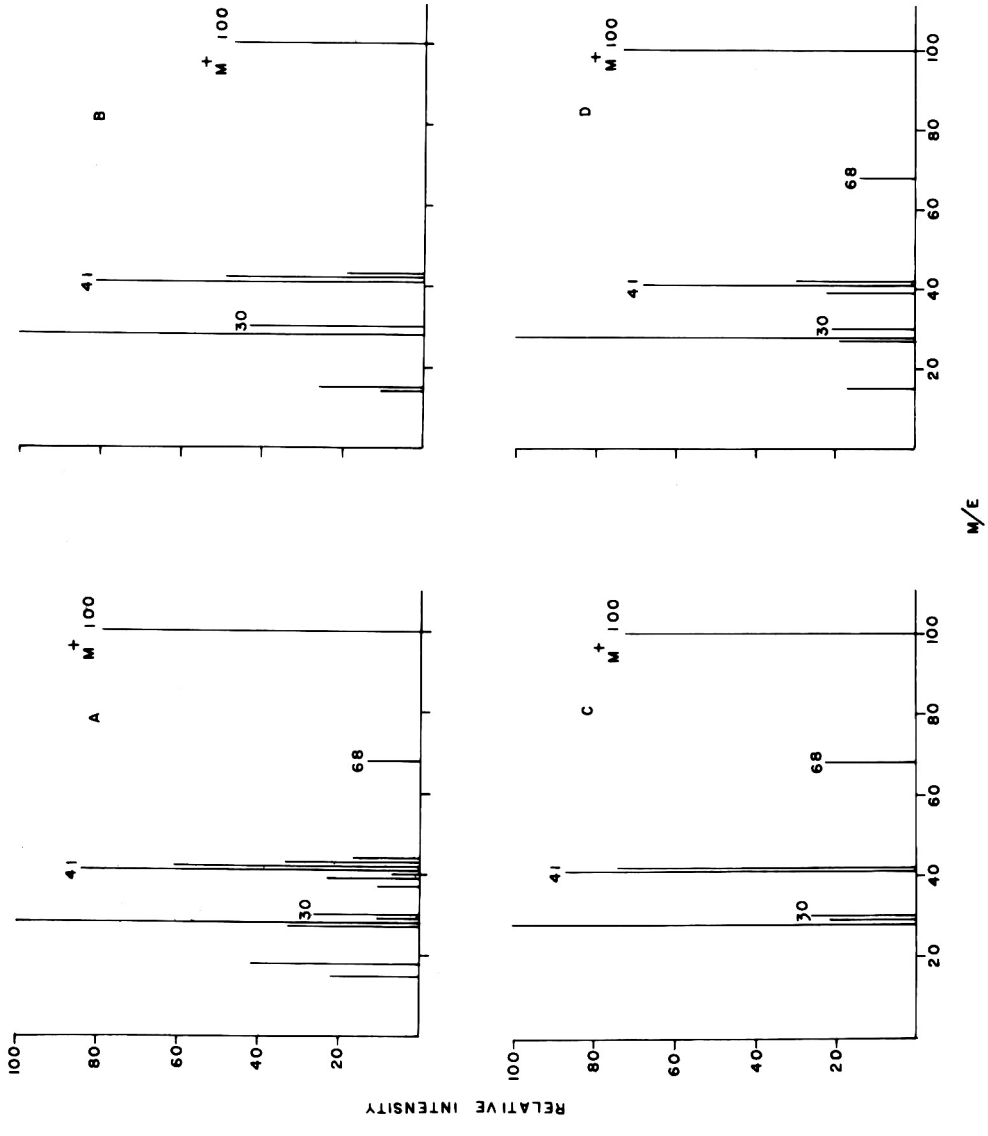

FIG. 4—Mass spectra of NPyr: A, standard; B, 1 μg/kg spiked cheese; C, 1 μg/kg spiked bacon; D, 2 μg/kg beef bacon.

Table 2. Volatile *N*-nitrosamine content of fried bacons

Brand	Concentration,[a] μg/kg	
	NDMA	NPyr
A	0.5	4[b]
B	5[b]	41
C	5[b]	84
D	2	4[b]
E	3[b]	17
F	0.5	2[b]

[a] Uncorrected for recovery.
[b] Confirmed by GC-MS.

NDMA spectra was the parent ion at m/z 74, and diagnostic ions m/z 42 and 30 were also present. All NPyr spectra gave intense parent ions at m/z 100 as well as diagnostic ions at m/z 41 and 30. These spectra may be taken as confirming qualitative evidence for the presence of these compounds.

Six fried commercial bacons analyzed by this procedure gave average NDMA and NPyr contents of 2.7 and 25 μg/kg, uncorrected for recovery (Table 2). These values may not represent current bacon manufacturing procedures. The lowest sample was a beef bacon-like product with 0.5 and 2 μg NDMA and NPyr/kg, respectively, and the highest NA content was 84 μg/kg for NPyr in bacon Sample C. A beef bacon-like product has previously been reported not to contain NPyr (13).

Several of those bacons which had NA contents at or below 5 μg/kg were confirmed by low resolution GC-MS to demonstrate the procedure. Representative spectra are given in Figs 3 and 4. Spectrum D in Fig. 3 is NDMA from bacon assayed at 3 μg/kg and spectrum D in Fig. 4 is NPyr from the beef bacon product. These spectra are typical of the GC-MS data generated at these levels. These spectra contain the diagnostic ions for these NAs and can be interpreted as being confirmatory qualitative evidence.

Three criteria must be met in order to confirm a volatile NA by our method. First, the unknown compound must be TEA-positive. Because of the high selectivity of the TEA, the vast majority of compounds are eliminated at this point. Second, the TEA-positive compound must give the correct retention time on both a packed and a capillary column. Good agreement on both columns was obtained for all samples analyzed in this study. Finally, the complete, full scan low resolution mass spectrum of the unknown must closely match the spectrum of the known NA.

Acknowledgment

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DRUGS

High Pressure Liquid Chromatographic Separation and Identification of Estrogens

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The use of high pressure liquid chromatography for separating mixtures of various estrogens is described. Separations of as many as 9 estrogens were studied using normal and reverse phase partition systems; the reverse phase system was the most useful. The estrogens were detected at 254 nm, a wavelength that favors their detection at low concentrations. The results of the separation studies were applied successfully to the separation and identification of estrogens in aqueous suspensions of estrogenic substances and to conjugated estrogens in tablets, following hydrolysis of the conjugates. Most estrogen peaks were identified using chromatographic mobilities; in some cases chemical reaction with the estrogen was used to form derivatives to aid identification. In one instance, the ultraviolet-absorbing properties of the estrogen were compared with literature data in order to assign an identity.

Estrogens constitute an important class of drugs. In addition to individual compounds that are used as therapeutic agents, complex mixtures such as an aqueous suspension of estrogenic substances and conjugated estrogens are useful. Several studies of the separation of estrogens using high pressure liquid chromatography (HPLC) have been reported. Tscherne and Capitano (1) separated 4 estrogens using silver nitrate in the mobile phase to interact with non-conjugated double bonds. In a study of steroid separations, Siggia and Dishman (2) included 13 estrogen compounds, 5 of which had some pharmaceutical significance. Butterfield *et al.* (3) described the mobilities of a number of equine estrogens, using several columns and mobile phases. Roos (4) studied the mobilities of equine estrogens and some estrogen derivatives and described a procedure for the quantitative analysis of conjugated estrogens. Krol *et al.* (5) used 2 columns in series to separate equine estrogens, and fluorescence detection. The separation of estrogen metabolites and conjugates has also been described (6–8). Recently, Roos (9) reported the determination of estrogens in pharmaceutical products by using the estrogen dansyl derivatives.

This work involves a study of the chromatographic behavior of 19 different estrogens with 5 columns and 6 different mobile phases. On the basis of these results, it is possible to detect and identify individual estrogens and the estrogens in a mixture of conjugated estrogens.

This paper was presented in part at the 92nd Annual Meeting of the AOAC, Oct. 16–19, 1978, at Washington, DC.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—DuPont Model 841 (E.I. du Pont de Nemours and Co., Wilmington, DE 19898) equipped with 254 nm ultraviolet (UV) detector.

(b) *Columns.*—μBondapak C_{18}, 300 × 3.9 mm (Waters Associates, Milford, MA 01757); Zorbax CN, 250 × 4.6 mm (E.I. du Pont de Nemours); Partisil PAC, 250 × 4.6 mm (Whatman Inc., Clifton, NJ 07014); μBondapak NH_2, 300 × 3.9 mm (Waters Associates); LiChrosorb Si 60 (5 μm), 250 × 3.2 mm (Altex Scientific, Berkeley, CA 94710).

(c) *Mobile phases.*—ACS certified grade, or equivalent, solvents were used.

(d) *Standards.*—All standard materials were of laboratory working grade.

Preparation of Estrogen Standard Solutions

Classical Estrogen and α-Estradiol Mixture.—Accurately weigh and quantitatively transfer to 25 mL glass-stoppered flask 13.952 mg estrone, 6.189 mg estradiol, 0.6031 mg α-estradiol, and 2.801 mg estriol. Dissolve in 6 mL methanol with heating. Let cool to room temperature.

Estradiol and Related Compounds.—Weigh and transfer to 50 mL glass-stoppered flask ca 50 mg each of estradiol, ethinyl estradiol, mestranol, estradiol valerate, estradiol dipropionate, estradiol cypionate, and 10 mg estradiol benzoate. Dissolve in 20 mL methanol with heating. Let cool to room temperature.

Equine Estrogen Mixture.—Accurately weigh and quantitatively transfer to 25 mL glass-stoppered flask 14.09 mg estrone, 6.928 mg equilin, 0.5198 mg equilenin, 5.132 mg α-estradiol, 4.986 mg estradiol, 5.279 mg α-dihydroequilim, 5.117

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mg β-dihydroequilin, 0.2261 mg α-dihydroequilenin, and 0.331 mg β-dihydroequilenin. Dissolve in 6 mL 2-propanol with heating. Let cool to room temperature.

Preparation of Solutions of Estrogens from Pharmaceutical Dosage Forms

Estrogenic Substances from Commercial Aqueous Suspension.—Transfer 10 mL aqueous suspension (5 mg/mL) to 50 mL centrifuge tube, add 30 mL water, shake, and centrifuge 10 min at 2000 rpm. Decant upper aqueous phase and discard. Add 30 mL water to centrifuge tube, shake, centrifuge as above, and discard upper aqueous layer. Add 10 mL methanol, heat on steam bath to dissolve residue, cool, and filter.

Estrogens from Conjugated Estrogen Tablet Dosage Form.—To remove tablet coating color, wash 10 tablets (2.5 mg/tablet) with water until wash is clear; then repeat washing process with acetone, vacuum dry, and finely powder dry, washed tablets. Transfer powder to beaker containing 6 g Celite, dry mix, add 4 mL water, mix, and pack into conventional 25 × 150 mm glass chromatographic tube containing glass wool plug. Pour 100 mL water-saturated $CHCl_3$ onto column and discard eluate. Add 10 mL $CHCl_3$ containing 150 mg dicyclohexylamine acetate to column, and elute with 100 mL $CHCl_3$, collecting eluate in 250 mL glass-stoppered flask. Evaporate eluate to dryness on steam bath, add 25 mL methanol, 1 mL HCl, and boiling chips, and gently boil 5 min. Cool, transfer to 250 mL separatory funnel with aid of 60 mL 6.5% KOH, and mix. Wash solution with two 60 mL portions of carbon tetrachloride and discard washings. Acidify with aqueous H_2SO_4 (1+2), cool, add 75 mL benzene, shake, and discard aqueous phase. Wash benzene successively with 10 mL water, six 10 mL portions of aqueous sodium carbonate (1+49), and two 10 mL portions of water; discard aqueous extracts. Pass benzene layer through glass chromatographic tube (25 × 150 mm) containing 30 g anhydrous Na_2SO_4 and collect eluate in 400 mL beaker. Wash Na_2SO_4 column with 25 mL benzene, collecting wash in 400 mL beaker. Evaporate to dryness on steam bath with aid of air current. Add 3 mL 2-propanol, warm to dissolve, cool, and transfer to 10 mL glass-stoppered flask.

High Pressure Liquid Chromatography

Conduct all chromatographic separations at ambient temperature with detection at 254 nm. Using conditions and settings described in appropriate figure caption, inject 10 μL solution into liquid chromatograph. Use estrone as internal standard with relative retention time of 1.00 and determine relative retention time for each peak. Identify various peaks from known relative retention times for estrogens observed for system under study. Since column variation may occur, inject known compound for chromatographic comparison to corroborate identity.

Results and Discussion

In this laboratory various estrogens are usually detected at 1 of 2 UV wavelengths. Estrogens characterized by unsaturation in conjugation with the benzene ring, such as 6-dehydroestrone and equilenin, have a more intense absorption at 254 nm than at 280 nm. On the other hand, estrogens containing no conjugation with the benzene ring, such as estrone, estradiol, and equilin, have stronger absorption properties at 280 nm, with an A_{280}/A_{254} ratio of about 7:1. In this work, separations are reported using detection at 254 nm in order to ensure detection of minor components in the mixture.

The chromatographic behavior of 19 estrogens is summarized in Table 1. Data are presented for a variety of columns and mobile phases, using estrone for relative retention times. Examination of the data shows that individual estrogens can be identified using a minimum of 2 columns. A number of the estrogen separations are represented in Table 1. In this work the μBondapak C_{18} column with acetonitrile–water mobile phases (A and B) and the Partisil PAC column with a propanol–cyclohexane mobile phase (F) were most useful. The Zorbax CN, μBondapak NH_2, and LiChrosorb Si 60 columns reported in Table 1 were not used further in this study.

In previous work (1) the classical estrogens (estriol, estradiol, and estrone) were separated using a μBondapak C_{18} column and methanol–water as the mobile phase. This separation is to be expected from the data in Table 1 using mobile phase C. Figure 1 shows the complete resolution of the 3 classical estrogens as well as α-estradiol. The ratio of the concentrations of estriol, estradiol, and estrone in this synthetic mixture is similar to what one might encounter in a tablet dosage form; a small amount of α-estradiol was added to demonstrate its separability. In the separation seen in Fig. 1, mobile phase A (Table 1) is used, with a slight (3%) increase in the acetonitrile concentration to provide for more rapid migration. The separation shown in Fig. 1 has a number of advantages compared with the separation

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Table 1. Relative retention times[a] of estrogens

Estrogen	μBondapak C_{18}				Zorbax CN		Partisil PAC	μBondapak NH_2	LiChrosorb Si 60
	A[b]	B[b]	C[b]	D[b]	E[b]	F[b]	F[b]	F[b]	F[b]
Estriol	0.20	—[c]	0.42	—	0.27	—	—	—	—
β-Dihydroequilenin	0.51	—	0.85	0.83	0.66	1.54	3.32	3.56	1.68
α-Dihydroequilenin	0.58	—	0.85	0.83	0.75	1.66	3.52	3.88	1.45
β-Dihydroequilin	0.59	—	0.99	0.70	0.66	1.10	2.14	2.06	1.60
6-Dehydroestradiol	0.63	—	—	—	—	1.14	2.03	—	—
α-Dihydroequilin	0.68	—	0.99	0.70	0.70	0.75	1.13	2.29	1.42
Estradiol	0.71	0.85	1.16	1.12	0.72	0.98	1.72	1.66	1.47
α-Estradiol	0.79	—	1.16	1.12	0.81	0.99	1.76	1.71	1.32
Equilenin	0.85	—	0.85	0.83	0.99	1.32	1.43	1.85	1.13
Isoequilenin	0.89	—	—	—	—	—	—	—	—
Ethinyl estradiol	0.89	0.88	—	—	—	1.07	1.65	—	1.14
Equilin	0.90	—	0.92	0.73	0.95	1.02	1.10	1.16	1.06
6-Dehydroestrone	0.93	—	—	—	—	1.03	1.14	—	—
Estrone	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mestranol	—	1.73	—	—	—	0.48	0.75	—	0.77
Estradiol benzoate	—	2.94	—	—	—	0.51	0.65	—	0.89
Estradiol valerate	—	3.51	—	—	—	0.57	0.58	—	0.68
Estradiol dipropionate	—	5.54	—	—	—	—	—	—	—
Estradiol cypionate	—	6.72	—	—	—	0.53	0.55	—	0.64
Estrone emergence, min	70.1	6.50	23.3	27.4	30.9	20.5	9.84	13.4	5.52
Pressure at inlet, psig	2000	1000	980	1500	1000	1000	1000	1500	2000
Flow rate, mL/min	1.26	1.10	0.50	0.60	0.65	0.77	1.17	1.25	1.15

[a] Retention times are expressed relative to estrone as 1.00.
[b] Mobile phase: A, 32% acetonitrile in water; B, 70% acetonitrile in water; C, 62% methanol in water; D, 62% methanol in water containing 1% silver nitrate; E, 35% acetonitrile in water; F, 5% 2-propanol in cyclohexane.
[c] Analysis was not performed.

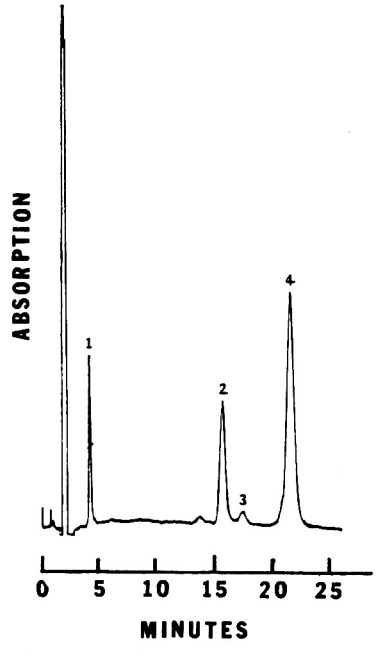

FIG. 1—Chromatographic separation of classical estrogens and α-estradiol. Column μBondapak C_{18}; mobile phase 35% acetonitrile in water; pressure at inlet 1500 psig; flow rate 1.35 mL/min; sensitivity 0.08 absorbance unit full scale (AUFS). 1, estriol; 2, estradiol; 3, α-estradiol; 4, estrone.

achieved by Tscherne and Capitano (1). First, the greater mobility of estradiol results in a sharper peak; second, estradiol is completely resolved from its isomer, α-estradiol; and third, an unidentified impurity, possibly 6- or 9-dehydroestradiol, with a retention time of about 14 min, is noted. This impurity elutes with estrone when the methanol-water mobile phase is used.

Table 1 indicates that estradiol and 4 of its ester derivatives are separated on several chromatographic columns, with elution order changes obtained between reverse and normal phase systems. Estradiol (peak 2, Fig. 2) elutes early relative to its 4 ester derivatives (peaks 7–10); it should be possible to detect this estrogen as an impurity in the derivative compounds. In a separation not shown here, estradiol valerate was spiked with estradiol at the 1% level, the level of the USP XIX (10) limit, and estradiol was detected without difficulty using the same system as in Fig. 2. This separation is also useful for the detection of ethinyl estradiol as an impurity in mestranol. Although estradiol and ethinyl estradiol elute almost together using the 70% acetonitrile in water mobile phase, the 2 estrogens are well

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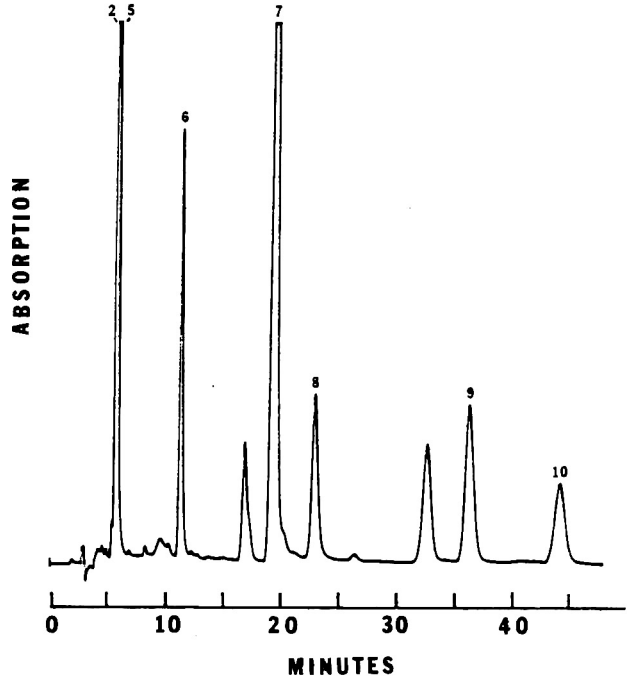

FIG. 2—Chromatographic separation of estradiol and related estrogens. Column μBondapak C_{18}; mobile phase 70% acetonitrile in water; pressure at inlet 1000 psig; flow rate 1.10 mL/min; sensitivity 0.04 AUFS. 2, estradiol; 5, ethinyl estradiol; 6, mestranol; 7, estradiol benzoate; 8, estradiol valerate; 9, estradiol dipropionate; 10, estradiol cypionate. The unidentified peaks at about 17 and 32 min are found in estradiol dipropionate.

separated using a 32% acetonitrile in water mobile phase (Table 1 relative retention times are estradiol 0.71 and ethinyl estradiol 0.89).

Figure 3 shows the separation of a mixture of 9 equine estrogens on a μBondapak C_{18} column, using acetonitrile–water and methanol–water mobile phases. The acetonitrile–water separation of estrogen carbonyls, i.e., equilenin, equilin, and estrone, is excellent in that near baseline separation has been achieved. The estrogen diol epimers are well resolved, with the β-isomer eluting before the α-isomer. However, α-dihydroequilenin and β-dihydroequilin elute together as a single, unresolved peak. The methanol–water mobile phase is not as useful as the acetonitrile system because a number of estrogens elute together; for example, the estrogen diol epimers are not separated.

The addition of silver ion to the methanol–water mobile phase (Table 1, mobile phase D) produces a different chromatographic mobility for only 3 of the estrogens, β-dihydroequilin (peak 13), α-dihydroequilin (peak 14), and equilin (peak 16). These estrogens, which are characterized by a double bond in the 7,8 position in ring B, exhibit greater mobility. This observation is the same as that of Tscherne and Capitano (1). Thus, although the chromatographic response to the presence of silver ion confirms that the double bond is exocyclic to ring A and not in conjugation with ring A, the value of the silver ion is limited.

A chromatogram of an extract of a commercial aqueous suspension of estrogens is shown in Fig. 4. This separation demonstrates the detection of estrone, the major component, and 2 minor components, equilenin and equilin, present at much lower levels. The shoulder on the estrone peak is unknown but is thought to be 9-dehydroestrone, a compound that absorbs much more strongly at 254 nm than does estrone. No other estrogenic substances are found here. The chromatographic system defined in Fig. 4 would be useful in the analysis of this dosage form because it can detect low levels of both equilin and equilenin and can separate these materials from estrone. Although the manufacturer claimed that traces of estrogen diols were present, no evidence of these materials was found (Fig. 4).

Figure 5 shows the separation of the estro-

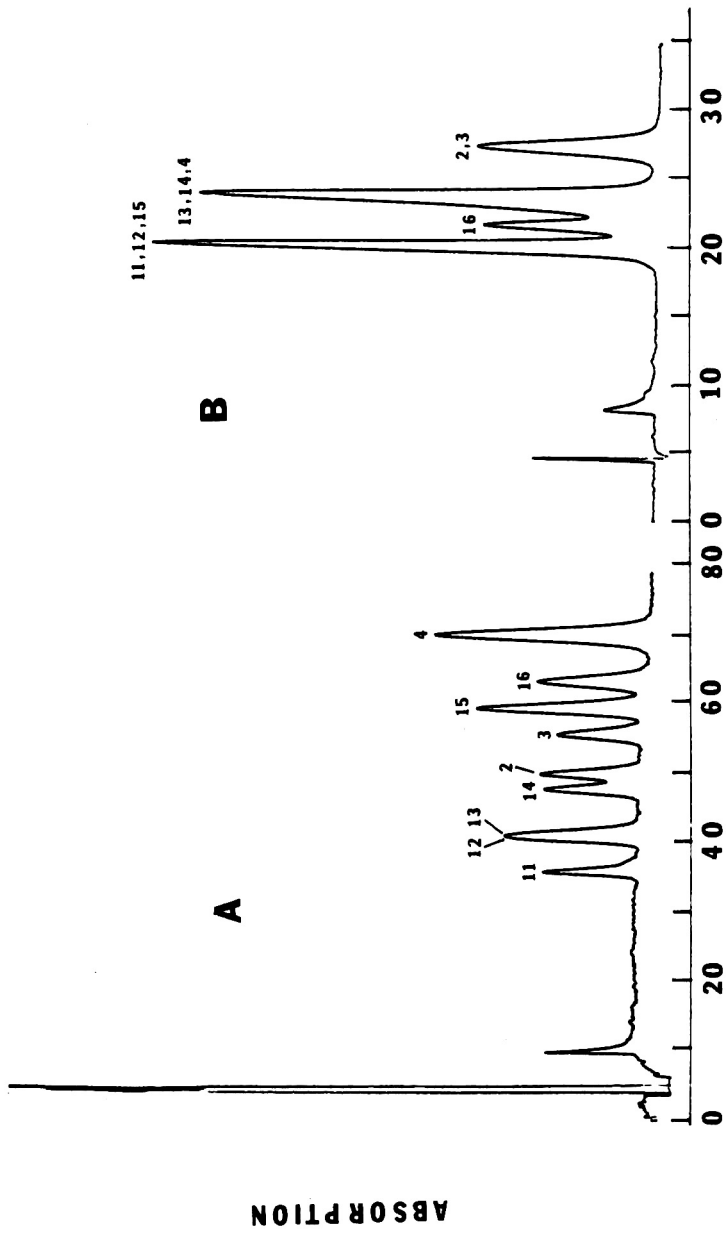

FIG. 3—Chromatographic separation of equine estrogens. Column μBondapak C_{18}; sensitivity 0.04 AUFS. 2, estradiol; 3, α-estradiol; 4, estrone; 11, β-dihydroequilenin; 12, α-dihydroequilenin; 13, β-dihydroequilin; 14, α-dihydroequilin; 15, equilenin; 16, equilin. A: mobile phase 32% acetonitrile in water; pressure at inlet 1200 psig; flow rate 1.26 mL/min. B: mobile phase 62% methanol in water; pressure at inlet 980 psig; flow rate 0.50 mL/min.

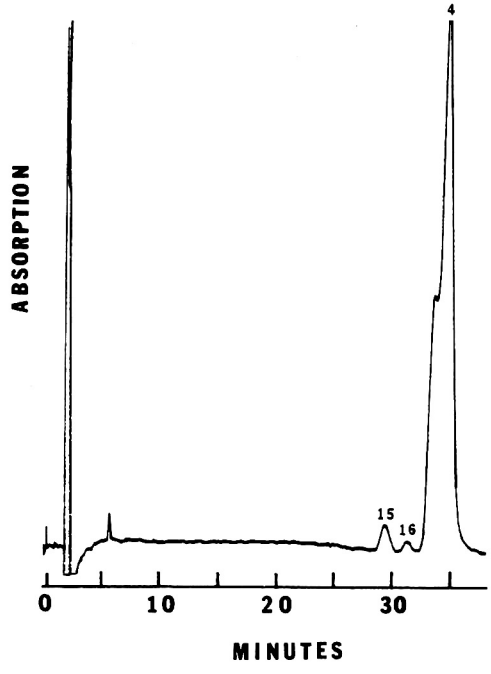

FIG. 4—Chromatogram of extract of commercial aqueous suspension of estrogenic substances. Column μBondapak C_{18}; mobile phase 35% acetonitrile in water; pressure at inlet 1500 psig; flow rate 0.90 mL/min; sensitivity 0.08 AUFS. 4, estrone; 15, equilenin; 16, equilin.

gens in an extract of a commercial conjugated estrogen tablet dosage form. The AOAC acid procedure (11) was used for hydrolysis. As expected, the appearance of the chromatograms is more complex than that from a standard mixture. On the basis of chromatographic mobility data, as many as 9 known compounds may be present; in addition, 4 other peaks are thought to be estrogenic materials. A number of unlabeled peaks that were observed and cannot be identified as estrogenic steroids may be dosage form constituents or phenolic materials, e.g., *o*-cresol, *p*-ethylphenol, as have been identified in another natural estrogen study using gas chromatography (12).

The numbered peaks in Fig. 5 were identified on the basis of 2 considerations. First, the estrogenic materials were divided into carbonyl and non-carbonyl compounds by using Girard-T reagent. The estrogens that correspond to peaks 15, 16, 19, 20, and 4 reacted with Girard-T reagent to form a derivative. The derivative was hydrolyzed, and the hydrolysate was then rechromatographed. The generated estrogens produced the same pattern, indicating the carbonyl nature of each compound. Generally, 4 estrogens with a carbonyl function (peaks 15, 16, 20, and 4) are found in conjugated estrogens (12, 13); in this study, the chromatographic systems have detected a fifth carbonyl compound (peak 19).

The second consideration was the comparison of mobilities of the estrogens with the mobilities of known compounds in a standard mixture. When the mobility of known compounds on the column used to record Fig. 5A were compared with the mobilities listed in Table 1 for μBondapak C_{18} (solvent A), the estrogen diol behavior was somewhat different. This experience indicates that relative retention times cannot be used as an absolute guide because variations do occur when 2 columns with the same stationary phase and the same mobile phase are used. For example, compounds 3 and 15 were separated (Fig. 3A); however, the separation was not reproduced by the same system as is observed in Fig. 5A. Seven compounds, 11, 12, 14, 2, 15, 16, and 4, were confirmed by comparison with a standard mixture. The identity of peaks 13 and 3 has been corroborated by the use of dansyl derivatives (Roos, R. W., 1979, unpublished data).

Two of the carbonyl compounds, 19 and 20, are unknown. Compound 20 is assumed to be 9-dehydroestrone because the chromatographic mobility of compound 20 was the same as that of the largest peak observed when the products of the isomerization of equilin with acid, carried out as described by Banes *et al.* (14), were chromatographed. The 2 largest peaks were collected and their UV spectra were determined. The UV absorption maxima and absorption spectrum shapes for compounds correspond to the data reported by Banes *et al.* (14), indicating that the 2 compounds are 9-dehydro-14-isoestrone and 8-dehydro-14-isoestrone; Banes *et al.* (14) assigned both compounds to the 14-iso series because heating in an acetic acid-hydrochloric acid mixture with some air oxidation converted both compounds to 14-isoequilenin. Roman *et al.* (12) have shown that the designation of these compounds as the 14-iso epimers (14) is incorrect; in addition, they indicate that the 9-dehydro compound reported is actually the 8-dehydro compound. Although the contention that the iso designation is incorrect seems valid, results

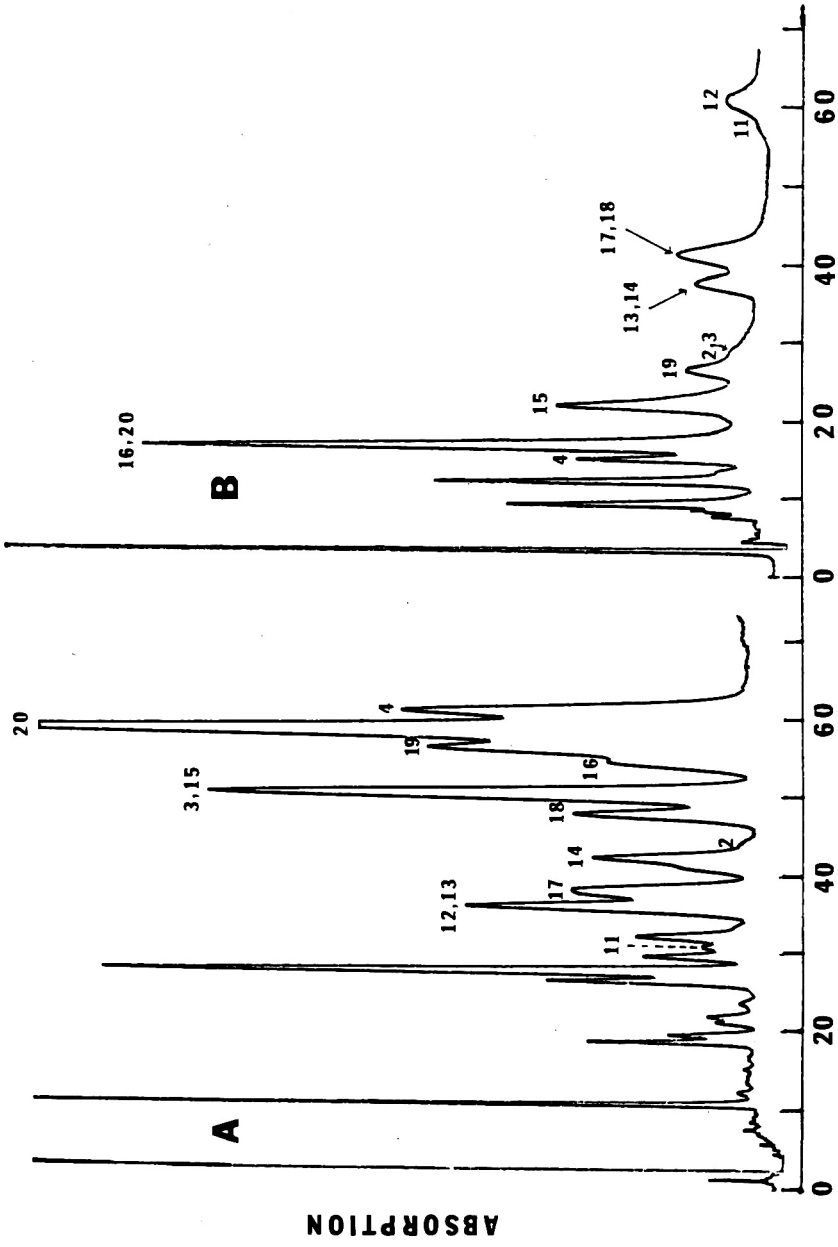

FIG. 5—Chromatograms of extract of equine estrogen from conjugated estrogen tablet. *See* Fig. 3 for identification of peaks 2-4 and 11-16; 17, 9-dehydroestradiol; 18, 9-dehydro-α-estradiol; 19, unidentified estrogen carbonyl; 20, 9-dehydroestrone. A: reverse phase column μBondapak C_{18}; mobile phase 32% acetonitrile in water; pressure at inlet 1400 psig; flow rate 1.06 mL/min; sensitivity 0.04 AUFS. B: normal phase column Partisil PAC; mobile phase 4.0% 2-propanol in cyclohexane; pressure at inlet 1500 psig; flow rate 1.75 mL/min; sensitivity 0.08 AUFS.

from our laboratory corroborate the Banes *et al.* (14) assignments of double bond position. The double bond position assignment is made here on the basis of the UV absorption at 280 nm. The UV absorption spectrum of 9-dehydroestrone at 280 nm decreases very rapidly compared to 8-dehydroestrone. This behavior is identical to that of 9-dehydroestrone in a commercial conjugated estrogen analyzed by HPLC with detection at 280 nm.

The identities of compounds 17, 18, and 19 have been tentatively assigned. The reduction of compound 20 results in compound 17; on the basis of accepting compound 20 as 9-dehydroestrone, compound 17 is assumed to be 9-dehydroestradiol. Use of reduction as an aid to estrogen identification has been discussed (15). The assignment of peak 18 as 9-dehydro-α-estradiol has been corroborated by use of dansyl derivative studies (Roos, R. W., 1979, unpublished data). Since the reduction product of peak 20 is peak 17, the elution of peaks 17 and 18 together (Fig. 5B) would establish their epimeric relationship. Although the compound contributing to peak 19 has not been identified, its position after equilenin (Fig. 5B) would suggest a similar structure, viz, aromatic A,B rings.

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Colorimetric Determination of Acetylenic Hypnotics by Formation of Silver Acetylides

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A colorimetric method is described for the determination of certain mono-substituted acetylenic hypnotic drugs—ethinamate, methyl pentynol carbamate, and ethchlorvynol. Silver acetylide is formed and extracted into isobutyl methyl ketone. After acidification, an equivalent amount of silver ions is liberated which is assayed colorimetrically as silver dithizonate (λ max. 472 nm). About 10–50 μg compound could be assayed with good accuracy. The procedure is reproducible and precise (standard deviation 1.5–2.19%). The procedure was successfully applied to different pharmaceutical dosage forms.

Ethchlorvynol, ethinamate, and methylpentynol carbamate are widely used as hypnotic and sedative drugs. These compounds are mono-substituted acetylene derivatives; the ethinyl group influences the drug activity (1). Because it was reported that such compounds are more effective and less toxic than their olefinic or saturated analogs, an analytical procedure based on this functional group is of considerable interest. Various procedures have been published for the determination of these compounds. Colorimetric (2), nuclear magnetic resonance (3), and gas-liquid chromatographic methods (4–8) are generally used for their determination in urine, biological fluids, or pharmaceutical dosage forms.

For the specific determination of mono-substituted acetylene derivatives, the simplest possibility is a method based on their reaction with metals, particularly with silver to form silver acetylide plus an equivalent of hydrogen ions (9). This reaction has been used in a variety of indirect titrimetric procedures (10, 11) and is specified in national pharmacopeias for the acidimetric determination of ethchlorvynol and ethinamate (12, 13). Measuring the silver adduct or excess silver ions is not as easy because a silver nitrate-silver acetylide complex with variable stoichiometric ratios tends to form under neutral or acidic conditions (9). In the presence of ammonia, however, the reaction is straightforward:

$$R-C\equiv CH + Ag(NH_3)^+_2 + OH^- \rightarrow R-C\equiv C\cdot Ag + H_2O + 2NH_3$$

This reaction had been previously used by Rizk *et al.* (14, 15) for estimating ethinyl steroids. The silver acetylide was either filtered or centrifuged and the excess silver ions were determined colorimetrically as dithizonate (14) or the silver acetylide was extracted into chloroform–benzene and the combined silver ion was estimated colorimetrically (15). Recently, this reaction was described for the atomic absorption spectroscopic estimation of phenylacetylene (16). It is based on the same principle as our previous work (14, 15): the silver acetylide formed is extracted into isobutyl methyl ketone and then acidified to liberate an equivalent amount of silver ion which is assayed colorimetrically as dithizonate.

This procedure permits a microdetermination of these compounds; as little as 10 μg can be assayed with good accuracy. Moreover, the procedure has been successfully applied to the determination of the acetylenic hypnotics in various pharmaceutical preparations.

METHODS

Apparatus and Reagents

(a) *Spectrometer.*—Pye-Unicam Sp. 600.

(b) *Silver nitrate solution.*—Accurately weigh 100 mg silver nitrate (AR), dissolve in 100 mL deionized water, and dilute an aliquot with water to contain 10 μg/mL.

(c) *Dithizone solution.*—0.004% (w/v) in $CHCl_3$.

(d) *Drugs.*—Ethchlorvynol (BP grade) was supplied by Abbott Laboratories Ltd, Queensborough, Kent, UK. Ethinamate (NF grade) was supplied by CID Laboratories, Giza, Egypt. Methylpentynol carbamate was supplied by Latèma Laboratories, 11, Bis. Rue Bolzae, Paris 8, France. Ethanolic solutions of each drug were prepared to contain ca 10–50 μg/mL.

Procedure

Calibration curve of $AgNO_3$.—Transfer to 5 separatory funnels volumes of silver nitrate solution equivalent to 10, 20, 30, 40, and 50 μg; add 10 mL water, 1 mL 2*M* H_2SO_4, and 5 mL $CHCl_3$. Shake vigorously between dropwise addition of dithizone solution from microburet; continue addi-

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tion and shaking until $CHCl_3$ layer containing yellow silver dithizonate acquires faint green color. This indicates complete extraction of silver ions. Dilute $CHCl_3$ extract to 10 mL with $CHCl_3$ in 10 mL volumetric flask, and measure absorbance at 472 nm against blank prepared by adding 2 drops of dithizone to 10 mL $CHCl_3$. Then plot calibration curve.

Assay of acetylenic hypnotic.—Transfer 1.0 mL aliquot of alcoholic solution of compound containing 10–50 μg to separatory funnel. Add 3 drops of 0.4% ammonia solution followed by 10 mL silver nitrate solution. Shake well, and let set ca 5 min. Extract silver acetylide with two 5 mL portions of isobutyl methyl ketone saturated with water. Collect organic extracts, and wash combined extracts with 3 mL water saturated with ketone. Extract organic layer with two 1 mL portions of 2*M* H_2SO_4. Dilute combined aqueous layers to ca 10 mL with water and add 5 mL $CHCl_3$. Complete as described above, starting with "Shake vigorously between dropwise addition of dithizone solution . . ." Determine content of silver nitrate and calculate content of drug in volume assayed. Each μg $AgNO_3$ is equivalent to 0.9841 μg ethinamate, 0.8510 μg ethchlorvynol, or 0.8310 μg methylpentynol carbamate.

Alternatively, assay drug by determining amount of silver nitrate remaining after extraction of silver acetylide by organic solvent, as described above.

Procedure for Pharmaceutical Preparation

Quantitatively transfer aliquot of powdered tablets, content of capsules (ethchlorvynol), or drops corresponding to ca 100 mg drug to 100 mL flask. Add 50 mL ethanol, shake thoroughly, dilute with ethanol, and filter if necessary. Transfer 20 mL of this solution to 100 mL volumetric flask and dilute to volume with ethanol. Then carry out assay for hypnotic acetylenes as previously described, using 1.0 mL diluted sample.

The content of the drug in its dosage form may also be determined by comparison with a standard curve prepared from different amounts of pure compound treated with silver nitrate. Record absorbance of extracted silver acetylide, assayed as dithizonate, and plot against concentration of pure drug.

Results and Discussion

Alkynyl silver compounds are insoluble in most organic solvents, apparently because of the formation of coordinate polymers (9). Silver acetylides of the ethinyl steroids, however, were solubilized in chloroform–benzene and were assayed colorimetrically as silver dithizonate (15). The solubility of silver acetylides of the hypnotics under investigation was tested in such a solvent mixture; only the silver acetylide of ethinamate was soluble. In contrast, isobutyl methyl ketone rapidly solubilizes these salts; however, because it also dissolves a certain amount of water, water-saturated solvent must be used for complete separation of the silver acetylides.

The calibration curves described above were consistently linear over the range 10–50 μg for silver ions and for the extracted silver acetylides.

Table 1 shows the results obtained on pure compounds. Different amounts are assayed (10, 20, and 30 μg) and the average per cent recovery is satisfactory. The per cent recovery is calculated either on the basis of silver ions extracted as silver acetylides or indirectly through the silver ions remaining in the aqueous layer. Thus the formation of the complex is consistently quantitative, and the silver acetylides are efficiently extracted by the organic solvents.

The precision and the reproducibility of the procedure was established from replicate determinations; the standard deviation ranged from 1.5 to 2.19%.

Table 2 shows the results for the drugs in different dosage forms by measurement of the extracted silver acetylides only. The drugs cannot be determined by measurement of excess silver ions because additives and excipients may

Table 1. Recovery (%) of acetylenic hypnotics[a]

Compound	10 μg		20 μg		30 μg		Official procedure
	Combined[b]	Excess[c]	Combined	Excess	Combined	Excess	
Ethinamate	98.2±2.1	99.0±1.9	96.8±1.5	101.2±2.02	100.8±1.62	98.6±2.19	101.5±1.6 (12)
Methylpentynol carbamate	96.0±1.7	102.1±1.9	100.2±1.9	97.9±2.07	98.9±1.68	100.2±1.89	99.9±1.3 (13)
Ethchlorvynol	100.3±1.62	98.6±2.3	98.8±1.7	97.8±1.92	98.4±1.72	100.5±2.1	99.6±1.2 (12)

[a] Mean % recovery of ≥3 determinations ± standard deviation.
[b] Recovery calculated on combined Ag^+ in organic layer.
[c] Recovery calculated on excess Ag^+ in aqueous layer.

Table 2. Determination of acetylenic hypnotic in various dosage forms[a]

Preparation	Labeled amt	Proposed procedure[b]		Official (12, 13)	
		Found	%	Found	%
Valamin tab. (Schering) (Ethinamate)	500 mg/tab	506.5 mg	101.3±1.52	504.5 mg	100.9±1.3
N. oblivon drops (Latèma) (methylpentynol carbamate)	20% w/v	19.92 w/v	99.6±1.63	20.04% w/v	100.2±1.0
N. oblivon tab. (Latèma) (methylpentynol carbamate)	100 mg/tab	103.5 mg	103.5±1.75	102.2 mg	102.2±1.1
Serenesil caps. (Abbott) (ethchlorvynol)	500 mg/cap	510 mg	102.0±1.5	501.5 mg	100.3±1.31

[a] Mean % recovery of ≥3 determinations ± standard deviation.
[b] The amount is calculated on the basis of acetylides extracted by the organic solvent.

interfere or consume some of the silver ions. The validity of the method for pharmaceutical preparations as well as the effect of interferences was demonstrated by assaying authentic samples containing the drug and common additives and excipients, e.g., lactose, magnesium stearate, and starch. The per cent recovery was satisfactory.

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High Pressure Liquid Chromatographic Determination of Antihistamine-Adrenergic Combination Products

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Reverse phase high pressure liquid chromatography (HPLC) with ion-pairing is used for the determination of pseudoephedrine hydrochloride in combination with triprolidine hydrochloride or chlorpheniramine maleate in sirups and tablets. Sirups require a preliminary column chromatography cleanup step. Response is linear for pseudoephedrine hydrochloride (range of 0–20 μg), chlorpheniramine maleate (range of 0–1.3 μg), and triprolidine hydrochloride (range of 0–1.0 μg). Recoveries from synthetic formulations were 98.8–101.3% for pseudoephedrine hydrochloride, 100.0–101.2% for chlorpheniramine maleate, and 97.7–99.8% for triprolidine hydrochloride. The coefficient of variation for the method is less than 1%.

Pseudoephedrine hydrochloride in combination with triprolidine hydrochloride or chlorpheniramine maleate is a commonly prescribed product for the symptomatic treatment of allergic rhinitis and vasomotor rhinitis. The decongestant effect of pseudoephedrine is combined with the antihistaminic properties of triprolidine or chlorpheniramine.

Antihistamine-adrenergic combination products have been analyzed using gas-liquid chromatography (GLC) (1) and column partition chromatographic separation followed by UV quantitation (2). HPLC circumvents problems associated with analysis of free amines by GLC because the amines are analyzed in their more stable salt forms. HPLC is also faster than methods requiring separate isolation and determinative steps.

Paired-ion reverse phase HPLC has become a widely used technique for the determination of both acidic and basic pharmaceutical dosage forms. The theory and applications have been reviewed (3–5). HPLC methods have been reported for several cough-cold preparations (6–9).

The purpose of this study was to develop a rapid and accurate method for the determination of combinations of pseudoephedrine with triprolidine or chlorpheniramine applicable to both sirups and solid dosage forms. A reverse phase HPLC system using an ion-pairing mobile solvent is employed. Diphenyldichlorosilane chemically bonded to microparticulate silica serves as the stationary phase. The mobile phase consists of 0.005*M* pentanesulfonic acid in an aqueous 35% acetonitrile–1% acetic acid solution.

Tablet sample preparation before HPLC requires dissolving the tablet material in water containing pheniramine maleate as an internal standard and then filtering. Sirup formulations require a preliminary column partition chromatographic cleanup to remove interfering components. A dilute sample solution containing internal standard is made basic, mixed with diatomaceous earth, and transferred to a short chromatographic tube. The amines are immediately eluted with water-saturated chloroform into a receiver containing alcoholic hydrochloric acid to reconvert the amines to their salt forms. Solvents are evaporated, and the resultant residue is dissolved in water and filtered for HPLC analysis.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Waters liquid chromatograph equipped with Model U6K injector, Model 6000A solvent delivery system, and Model 440 absorbance detector (Waters Associates, Inc., Milford, MA 01757), or equivalent. Operating conditions: Flow rate 1.5 mL/min; 254 nm detector 0.05 AUFS; temperature ambient; amount injected 10 μL.

(b) *HPLC column.*—μBondapak Phenyl, 10 μm particle size, 3.9 mm (id) × 30 cm long (Waters Associates), or equivalent. Wash column daily after use with water followed by methanol.

(c) *Recorder.*—10 mV with 0.5 cm/min chart speed (Omniscribe B-5000, Houston Instrument, Austin, TX 78753), or equivalent.

(d) *Integrator.*—Chromatopac-E1A data processor (Shimadzu Scientific Instruments, Inc., Columbia, MD), or equivalent.

(e) *Filters.*—Millipore types HA and FH (pore size 0.45 μm) and type AP prefilter (Millipore Corp., Bedford, MA 01730), or equivalent.

(f) *Chromatographic tubes.*—25 × 2.5 cm od, glass column with one restricted end.

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Reagents

(a) *Solvents.* — Distilled-in-glass (Burdick & Jackson Laboratories, Muskegon, MI 49442), or equivalent.

(b) *1.0N Sodium hydroxide–0.5N sodium chloride solution.*—Dilute 2.0 g NaOH and 1.46 g NaCl to 50 mL with water.

(c) *Diatomaceous earth.* — Celite 545, acid-washed (Johns-Manville Corp.), or equivalent.

(d) *Internal standard solution.*—0.4 mg pheniramine maleate/mL water.

(e) *Antihistamine solution.*—Transfer 31.25 mg accurately weighed Triprolidine Hydrochloride NF Reference Standard or 50 mg Chlorpheniramine Maleate USP Reference Standard to 100 mL volumetric flask and dilute to volume with water.

(f) *HPLC standard solution.*—Transfer 30 mg accurately weighed Pseudoephedrine Hydrochloride NF Reference Standard to 10 mL volumetric flask. Pipet 4.0 mL antihistamine solution and 5.0 mL internal standard solution into flask and dilute to volume with water. Transfer 2.0 mL aliquot and 2.0 mL water to small glass-stoppered flask and mix.

(g) *HPLC mobile phase.*—Transfer 1 bottle PIC Reagent B-5 (Waters Associates) to 1 L volumetric flask. Add 350 mL acetonitrile, and dilute to volume with water. Stir magnetically 5 min. Filter through type FH filter. Sonicate 10 min to deaerate. (PIC Reagent B-5 contains sufficient pentanesulfonic acid and acetic acid so that when 1 bottle is diluted to 1 L, their respective concentrations are 0.005*M* and 1%.)

Sample Preparation

(a) *Tablets.*—Weigh and finely powder 20 tablets. Transfer accurately weighed portion of the powder equivalent to 60 mg pseudoephedrine hydrochloride to 50 mL glass-stoppered flask. Pipet 10 mL internal standard solution and 30 mL water into flask and place in ultrasonic bath 1 min. Stopper and shake flask. Filter portion of solution through type HA filter into small glass-stoppered flask. Use type AP prefilter if necessary.

(b) *Sirups.*—Pipet 5.0 mL internal standard solution into 10 mL volumetric flask. Carefully add sample sirup to volume (do not wet flask above mark) and mix. Place small glass wool plug in base of chromatographic tube as support. Mix 0.7 mL 1.0*N* NaOH–0.5*N* NaCl solution and 1.0 g diatomaceous earth. Transfer mixture to tube and tamp to uniform mass. Pipet 2.0 mL aliquot diluted sample solution into a 100 mL beaker. Add 0.3 mL 1.0*N* NaOH–0.5*N* NaCl solution and swirl to mix. Add 3.5 g diatomaceous earth and mix thoroughly. Transfer mixture to tube in 3 portions, tamping after addition of each portion. Dry-wash beaker with 1.0 g diatomaceous earth, add to column, and tamp. Cover with small glass wool plug. Elute column with four 50 mL portions of water-saturated chloroform into a glass-stoppered 250 mL Erlenmeyer flask containing 5 mL alcohol and 5 drops of hydrochloric acid. Evaporate to dryness on steam bath with stream of air. Add 5 mL alcohol and evaporate to dryness again. Pipet 4.0 mL water into flask and swirl to dissolve residue. Filter solution through type HA filter into small glass-stoppered flask.

Determination

Let HPLC system equilibrate with column in instrument and set mobile phase at flow rate of 1.5 mL/min. Inject 10 μL HPLC standard solution. The 4 peaks (maleic acid, pseudoephedrine, pheniramine, and triprolidine or chlorpheniramine) should be completely resolved and symmetrical. Make replicate injections of HPLC standard solution and compare area response ratios relative to internal standard area response to ascertain reproducibility of system. Proceed with sample analysis, using three 10 μL injections each of sample and HPLC standard solutions. If an interfering peak (methylparaben) is observed in chromatogram of sirup sample, in region of pheniramine internal standard peak, repeat column chromatography cleanup, using another 2.0 mL aliquot of diluted sample solution. Pack column firmly.

Calculations

Calculate results, using area response ratios (RR) relative to internal standard:

$$\text{mg amine salt per tablet} = (RR_u/RR_s) \times mg_s \times (TW/SW) \times 2$$

$$\text{mg amine salt per 5 mL sirup} = (RR_u/RR_s) \times mg_s$$

where RR_u = sample area response ratio; RR_s = standard area response ratio; mg_s = mg amine salt in initial HPLC standard 10 mL dilution; TW = average tablet weight; SW = sample weight.

Results and Discussion

Recovery data were obtained for 5 pseudoephedrine hydrochloride-triprolidine hydrochloride synthetic formulations (2 sirups, 3 tablets) and 2 pseudoephedrine hydrochloride-chlorpheniramine maleate sirup synthetic formulations. Results are summarized in Table 1. Data obtained by the second analyst in Table 1 are the results of an intralaboratory study.

Repeatability of the method was determined for pseudoephedrine hydrochloride and triprolidine hydrochloride on the basis of 10 replicate assays of a synthetic sirup formulation. The coefficient of variation (CV) was 0.4%

Table 1. Recovery data (% recovery) for synthetic formulations[a]

Formulation	Dosage form	Pseudoephedrine HCl	Triprolidine HCl	Chlorpheniramine maleate
A	sirup	101.3	99.4	—
B	sirup	100.6[b] (98.9)	99.2[b](97.7)	—
C	sirup	101.0 (100.4)	—	101.2 (100.9)
D	sirup	98.8 (100.8)	—	100.0 (100.0)
E	tablet	99.9	99.6	—
F	tablet	100.5	99.8	—
G	tablet	99.3	99.4	—

[a] Values in parentheses obtained by second analyst.
[b] Average of 10 replicate assays.

for pseudoephedrine hydrochloride and 0.6% for triprolidine hydrochloride.

Linearity of area response ratios was observed for pseudoephedrine hydrochloride (range of 0–20 μg), chlorpheniramine maleate (range of 0–1.3 μg), and triprolidine hydrochloride (range of 0–1.0 μg).

The HPLC method was used to assay 11 pseudoephedrine hydrochloride-triprolidine hydrochloride sirups (representing 5 different manufacturers) and 3 different pseudoepherine hydrochloride-chlorpheniramine maleate sirups. Results are summarized in Table 2. Figure 1 shows typical chromatograms of a pseudoephedrine hydrochloride-triprolidine hydrochloride sirup analysis. Note the absence of maleic acid in sirup chromatogram due to sample cleanup. Pseudoephedrine hydrochloride-chlorpheniramine maleate chromatograms are similar, with chlorpheniramine having a retention time of 5.1 min.

Most of the commercial sirups declared 30 mg pseudoephedrine hydrochloride in combination with 1.25 mg triprolidine hydrochloride or 2 mg chlorpheniramine maleate per 5 mL. Tablet synthetic formulation recovery determinations were performed at the individual tablet dosage level of 60 mg pseudoephedrine hydrochloride and 2.5 mg triprolidine hydrochloride.

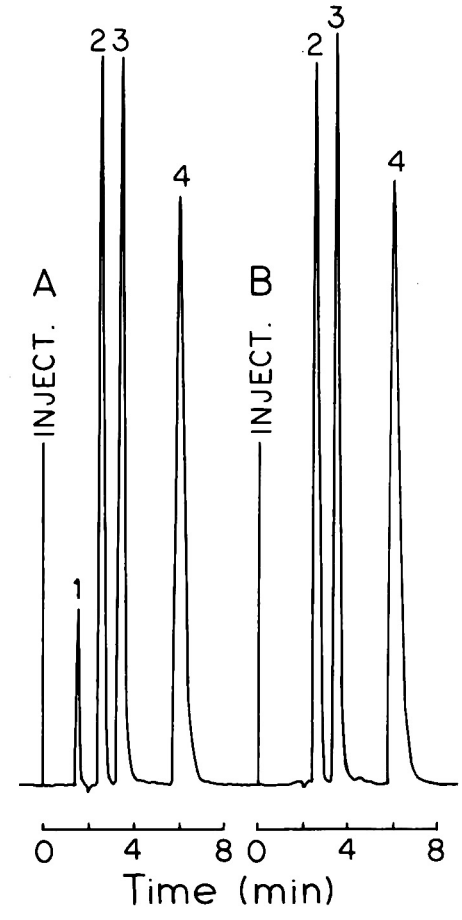

FIG. 1—Typical chromatograms of A, standard solution: 1, maleic acid; 2, pseudoephedrine; 3, pheniramine internal standard; 4, triprolidine; and B, psuedoephedrine hydrochloride-triprolidine hydrochloride sirup sample solution.

Table 2. Results of sirup assays (% declared)

Sirup	Manuf.	Pseudoephedrine HCl	Triprolidine HCl	Chlorpheniramine maleate
1	A	99.0	95.2	—
2	B	104.3	102.4	—
3	B	104.3	101.6	—
4	C	102.0	100.0	—
5	D	101.0	94.4	—
6	D	103.0	95.2	—
7	E	98.7	104.8	—
8	E	100.3	105.6	—
9	E	100.0	105.6	—
10	E	99.3	104.0	—
11	E	102.0	106.4	—
12	F	102.7	—	105.0
13	G	101.0	—	102.4
14	H	107.6	—	108.7

The method presents a rapid and accurate procedure for analyzing pseudoephedrine hydrochloride in combination with triprolidine hydrochloride or chlorpheniramine maleate with potential for application to other cough-cold preparations containing basic as well as neutral active components.

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REMINDER: Deadlines

Nominations for the 1979 Fellows of the AOAC Award—March 3, 1980

Nominations for the 1979 Harvey W. Wiley Award—April 1, 1980

Nominations for the 1979–1980 Scholarship Award—May 1, 1980

For further information and nomination forms for Wiley Award, contact AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209

MYCOTOXINS

Protocols for Surveys, Sampling, Post-Collection Handling, and Analysis of Grain Samples Involved in Mycotoxin Problems[1]

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This report examines and summarizes current knowledge regarding mycotoxin surveys, sampling techniques, conditions conducive to post-collection production of mycotoxins in grain samples, and analytical methods for mycotoxin analysis. Priority attention is given to samples of corn suspected of containing aflatoxin. The report includes recommendations where deemed appropriate by the Ad Hoc Work Group.

Surveys for Evaluating Mycotoxin Problems

General Comments

Surveys as used in this report means sampling efforts designed so that statistically sound inferences may be made concerning mycotoxins in a defined population of grain. The population may be grain in any stage of production: harvesting, transport, processing, or utilization; in any area: state, region, or nation; and for any particular time period.

Surveys to evaluate mycotoxin problems in corn have been conducted (1). It has become apparent that since many agencies (public, private, national, international, etc.) may conduct these surveys, it would be advantageous to have a set of guidelines.

[1] Report of the Ad Hoc Work Group to State Agricultural Experiment Stations-U.S. Department of Agriculture Mycotoxin Research Coordinating Committee.

Recommendations

Before a survey is made, the purpose of the survey should be carefully determined. If a survey is justified, only surveys based on probability sampling methodology should be used so that reliable inferences can be made. If possible, standard protocol should be followed as outlined elsewhere in this report. The population to be surveyed must be agreed upon at the outset. Certain questions must be considered based on inferences to be made from the survey. For example, in a survey of corn, should samples be taken of mature corn standing in the field or should samples be taken at some later stage, perhaps at time of marketing or before feeding? It depends on the results needed. If sampling is done in the field just before harvest, how many days before harvest can samples be gathered and still meet survey objectives? One must know at which stage aflatoxin forms in the field. These and other considerations must be addressed in defining the population of interest.

Once the population is specified, a suitable sampling frame to assess fields, farms, or bins must be identified or developed. The sampling frame must be able to assess sampling units with known probabilities.

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A randomization process has to be part of any field sampling procedure so selectivity in sampling and resulting biases can be avoided. A bin can be adequately sampled only while it is being loaded or unloaded. Some recent research has been carried out to study the components of variance associated with sampling and subsampling from a lot of shelled corn (2). Such research is needed in order to determine optimum field sampling procedure.

Handling and testing procedures should be standardized. Efforts to plan and carry out well designed surveys will be of little value if inconsistent procedures which produce noncomparable results are used. We hope that the best procedures can be agreed upon and used uniformly. Surveys not based on sound statistical design should not be authorized or conducted.

Sampling and Subsampling Techniques

General Comments

Surveys for mycotoxins in agricultural products sometimes are based on the misconception that the distribution of the survey samples according to mycotoxin concentration closely approximates the distribution of the lots which were sampled. One reason for lack of agreement between sample distributions and lot distributions is sampling error caused by inadequate sample size. Other reasons for error in surveys are biased sampling procedures, inadequate sample comminution, and improper subsampling for analysis. Because more is known about sampling for aflatoxin, causes of sampling error will be discussed in relation to aflatoxin, but the general concepts probably apply to other mycotoxins.

Sample Size

High concentrations of aflatoxin have been found in individual kernels of corn, peanuts, and cottonseed (3, 4, 5). These high concentrations probably occur in individual seeds, nuts, or fruits of other commodities which are susceptible to aflatoxin contamination. Because aflatoxin is often highly concentrated in but a small percentage of the seed within a lot, there is a large variation in the aflatoxin concentration of samples from the lot, and determination of the true aflatoxin concentration in the lot is difficult (2, 6–8). The variance of estimated concentrations is inversely proportional to sample size.

The distribution of sample concentrations about the true aflatoxin concentration in a lot is skewed so that more than half of the sample concentrations are less than the lot concentration (9). Skewness decreases as sample size increases, and the distribution will approach a normal distribution for large samples. As a result of this skewness, the distribution of sample concentrations determined in a survey will be different from the distribution of the true concentration in the lots sampled. More than half of the individual sample concentrations will be lower than the true aflatoxin concentration in the lot from which the sample was drawn, but some of the time the sample concentration will be much higher than the lot concentration. However, when the number and size of the samples are adequate, the average aflatoxin concentration in the samples will provide a useful estimate of the average aflatoxin concentration in the lots which were sampled.

It is difficult to make a general recommendation about the size samples (amount of grain per sample) that should be used in surveys. Larger samples will increase accuracy, but the cost of the product transportation, etc., may be limiting. Cost factors must be reconciled with acceptable limits of accuracy for the anticipated survey. The effect of sample size on the agreement between the sample distribution and the lot distribution may be determined according to a procedure developed by Whitaker and Dickens (10). This same procedure may be used to estimate the lot distribution based on the sample distribution.

If the purpose of the survey is limited to a determination of the average aflatoxin concentration in all lots sampled, relatively small samples are required. When an accurate estimate of the distribution of lots according to aflatoxin concentration is desired, larger samples are required. A 10 lb sample of shelled corn is probably sufficient for most survey purposes. For the same accuracy, much larger samples of cottonseed, shelled peanuts, and possibly other products are needed.

Sampling Procedures

Samples may be taken from crops growing in the field, during handling, during storage, and at other points in the production, marketing, and processing system. Each type of sampling presents a different situation regarding the distribution of the aflatoxin-contaminated seed

within the lot sampled and accessibility of the entire lot. When feasible, samples should be taken after the lot has been reduced to a smaller particulate size. For example, it is better to sample shelled corn rather than ear corn, and it is better to sample ground corn rather than shelled corn.

When the lot has been recently blended by harvesting, loading and unloading, turning, grinding, or other operations, a representative sample is more easily drawn than when the lot has not been blended because growth may have occurred in spots. For example, moisture condensation or leaks during storage may cause a portion of the lot to mold and contain high concentrations of aflatoxin. It is impossible to predict where to probe the contents of the storage bin in order to obtain a sample with the same aflatoxin concentration as the concentration in the entire lot.

Stream Sampling.—The most effective sampling method is to take small portions from a moving stream at periodic intervals and combine the portions into a sample. Cross-cut samplers are commercially available which automatically cut through the stream at predetermined intervals (11). When an automatic cross-cut sampler is not available, a person may be assigned to pass a cup through the stream at periodic intervals and thus collect a sample. The stream should be sampled frequently but the amount taken at each interval may be small to avoid accumulating too large a sample. The samples must be taken from the stream throughout the time the lot is moved. This is the only proper way to sample a bin.

Probe Sampling.—Probe sampling is probably adequate for lots which have recently been blended by harvesting or handling operations. Recommended methods for taking probe samples are published by the American Oil Chemists' Society (12). As previously stated, probe sampling of lots which have not been blended recently probably will not provide representative samples because mold growth may have occurred during storage if moist conditions are present.

Field Sampling.—When samples are taken before harvest, reliable sampling representative of the field is difficult. When *Aspergillus flavus* grows on corn before harvest, the geographic distribution of infected ears within the field may be erratic. Also, none of the kernels, a single kernel, a group of kernels, or nearly all of the kernels on an ear may be aflatoxin-contaminated. The problem becomes one of collecting a large number of widely distributed ears in order to obtain a representative sample of ears which must then be shelled so a representative sample of the shelled corn can be subdivided. Therefore, sampling should be coordinated with harvesting so corn from a very large number of ears is represented in the sample of shelled corn. The same concept applies to other commodities where the contaminated product may be clustered before harvest.

When it is absolutely necessary to collect samples of ears from the field, one must recognize the possible errors associated with such a procedure in estimating for the field. The coefficients of variation associated with aflatoxin test data when various numbers of ears are harvested for a sample were determined. The estimated coefficients of variation (%) among aflatoxin tests when the indicated number of ears of corn are randomly selected from a field with an average of 100 ppb aflatoxin are 133 for 36 ears harvested and shelled, 83 for 100 ears, 62 for 200 ears, 48 for 400 ears, 39 for 800 ears, and 29 for the entire field (unpublished data from J. W. Dickens). (After the ears are shelled, a 4.54 kg sample of kernels is comminuted and analyzed according to the AOAC procedure (13).) The estimated coefficient of variation associated with a 36-ear sample from a field with an average aflatoxin concentration of 100 ppb is 133% compared to 29% when the entire field is harvested and a 4.54 kg sample of kernels is ground to obtain the analytical sample. The estimate of 133% is based on the assumption that the ears are selected from the field in a completely random manner.

Subsampling

Since it is not feasible to extract the entire sample for aflatoxin analysis, the large sample must be comminuted so a subsample of the comminuted material can be extracted (13, 14). A larger subsample is required for coarsely ground material than for finely ground material. Recommended methods for comminution and subsampling are given by the AOAC (13).

Post-Collection Production of Mycotoxins

General Comments

In general, grain (corn) with moisture contents of 13–13.5% is safe for long term storage.

There are many examples to demonstrate the

rapidity with which fungi develop but 2 should suffice. A recent study (unpublished data from G. C. Kingsland) on the effect of grain moisture and 2 storage temperatures on growth of *Aspergillus flavus* as measured by an increase in the numbers of propagules/g corn substrate indicated dramatically the effect of these factors. For example, when corn was stored at 0° C for 8 days at 21.3% moisture, there was virtually no growth of the fungus. However, when a duplicate sample was stored at 24°C, propagules increased 1500-fold (from 278 propagules/g to 420,000 p/g). When another corn sample was stored at a moisture content of 17.2%, propagule number increased approximately 8-fold (from 40,000 to 320,000 p/g). At 12.6% moisture, there was virtually no growth (from 21,500 to 29,500 p/g) in another sample. Another study (unpublished data from D. M. Wilson) indicated an increase in aflatoxin content of approximately 10-fold (from 200 to 2300 ppb aflatoxin) in a 3-day period when a field-harvested corn sample was stored at high moisture.

Although most "storage" and "field" fungi, especially members of the genus *Aspergillus*, have rather precise minimum requirements of water activity (A_w), once these limits are breached rampant growth may occur. Also, the longer the time, the lower the A_w and temperature required to prevent fungus growth. Although mold presence is not evidence *per se* of aflatoxin or any other mycotoxin, mycotoxin production is not likely in the absence of mold. Average moisture contents may be misleading because they do not reflect the range among individual kernels. If the moisture and temperature at a spot in a grain sample are adequate, mold growth will occur and respiration of the mold and grain may increase the moisture content and temperature of that portion of the sample. Insect infestations that damage kernels may also contribute to fungal growth.

Corn harvested wet must be dried rapidly to prevent mold growth, i.e., to less than 13–13.5% moisture. Samples harvested dry must be kept dry. When moisture and temperature are favorable for mold growth, even for a very short period, significant production of mycotoxins can occur. Preferably, samples should be stored at 0°C, although economics generally preclude this practice in large surveys. Proper drying, as discussed above, is the most certain way of preventing formation of aflatoxin in samples of corn.

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Recommendations for Prevention and Control

Controlling the conditions of storage to prevent mold growth and mycotoxin elaboration from harvest to chemical analysis for mycotoxins is a very difficult problem for surveys involving a large number of samples from widely dispersed locations. Current information strongly indicates that in order to prevent mold growth and/or mycotoxin elaboration it is necessary to keep the sample dry or at 0°C or below. Analysis of the problem is simple, but a solution is not. The following procedures could minimize opportunities for mycotoxin production:

(1) Preferably, samples should be placed in transit in a dry condition and every effort should be made to maintain dryness.

(2) Plastic bags should not be used for unrefrigerated storage unless seeds are dead and dry.

(3) Closely packed piles of samples may not cool rapidly enough to 0°C under refrigeration. Therefore, the samples should be spread until they have been cooled. Since even the mass of 10 lb samples may retard cooling, sample bags should be large enough so the sample can be spread within the bag to facilitate rapid cooling.

(4) Once cooled to 0°C (Dry Ice, liquid nitrogen, or refrigeration) removal therefrom can quickly result in condensation and elevate the moisture availability to unacceptable levels. Therefore, samples under refrigeration either should be analyzed immediately upon removal or other special efforts should be taken to prevent condensation on the sample, e.g., keeping samples in moistureproof containers on removal from refrigeration until samples reach ambient temperatures.

(5) It may be possible to add toxicants such as acetic acid, propionic acid, etc., to a shipment before transit. There are several shortcomings to this procedure: the volume of the sample markedly influences the feasibility of this procedure; corrosive and toxic nature of such materials may represent a health hazard to personnel; this method would be lethal to all members of the microflora and thus identification of the contaminants would be impossible; and effects of such treatment on subsequent analytical procedures have not been determined. Thus, more

research is needed before such procedures can be recommended.

(6) Time in transit should be minimized.

Analytical Problems

Current Knowledge

The official first action method adopted by the AOAC (13) and the American Association of Cereal Chemists (AACC) (15) for determining aflatoxin in corn is known as the CB method. It involves extraction of the ground corn sample with chloroform in the presence of water. The extract is purified on a silica gel column for measurement of aflatoxins by thin layer chromatography.

The coefficient of variation for one analysis by the CB method is 33% among laboratories and 17% within a laboratory. The BF method developed for peanuts has not been tested collaboratively on corn, but the coefficient of variation is 27% within a laboratory and recoveries are 30–35%, too low to be quantitative (16). The Pons method developed for cottonseed and used in some laboratories for corn has a coefficient of variation of 31% on corn and recoveries greater than 90% for samples containing less than 100 ppb (16). One proposed method includes a purification of extracts by liquid-liquid partition (17), but recoveries are low. In another proposed method, the amount of aflatoxin in an extract is determined by measuring the enhancement of fluorescence in solution after the reaction of aflatoxin with iodine (18).

Surveys can be performed using rapid screening techniques to determine the presence or absence of aflatoxin (13), and quantitating positive samples by the CB method. Minicolumn techniques have been used in surveys to determine the incidence of aflatoxin in corn samples and the approximate levels of contamination in samples. A minicolumn method for aflatoxin in corn that includes the Holaday extraction and cleanup (19) and the Velasco column (9, 20) was approved in 1978 for adoption as official first action by the AOAC.

Aflatoxin identity can be confirmed in extracts by forming the water adduct on a TLC plate with trifluoroacetic acid (13). Virtually all substances in corn determined to be aflatoxin by the CB method have been confirmed to be aflatoxin by formation of the water adduct.

A method for determining zearalenone in corn has been adopted by both the AOAC and AACC (21, 22). The extraction solvent is chloroform in the presence of water, and extracts are purified for TLC on silica gel columns. A second method designed for zearalenone in grains and feeds includes a liquid-liquid partition into basic solution. The mycotoxin is measured in solution by gas-liquid chromatography (GLC) (23). The second method has been studied collaboratively, but the results have not been published. One can confirm results obtained for zearalenone by TLC with GLC and those obtained by GLC with TLC. Final confirmation of identity for zearalenone in a feed or grain is accomplished by GLC-mass spectrometry.

A method for determining ochratoxin in barley (13) has been adopted as well as one for sterigmatocystin in several grains (13).

Recommendations

Currently validated and officially adopted methods should be used in all surveys. Research should be encouraged to improve analytical methods for mycotoxins. Lower coefficients of variation and better recoveries are needed. New methods should be considered and tested collaboratively as they are developed. The disadvantages of adopted methods are that the quantities and types of solvents required make the methods expensive, and the solvents are hazardous and toxic. Mycotoxin analytical methods are needed that require less time, less solvent, and less toxic solvents without sacrificing reliability. Such methods must be tested collaboratively before recommendation and official adoption.

Mycotoxins for which reliable, validated, sensitive methods are not available are ochratoxin (corn), penicillic acid, citrinin, and the trichothecenes. Research is needed to develop adequate quantitative methods for these mycotoxins.

Needs and Recommendations

Research Needs

(a) *Sampling of a Corn Field.*—There is insufficient information on the distribution of ears according to aflatoxin concentration within a field of corn. The protocol recommended in this report should be tested and analyzed, and improvements should be made as necessary.

(b) *Sampling of Containers* (storage bins, truck loads, etc).—The only practical way to sample a bin is during loading and unloading.

The geometry of a bin and limited access to the bin and its contents make it difficult to obtain samples that reflect both the distribution and the mean concentration of aflatoxin in a bin. New sampling designs and equipment for obtaining samples would be of great value.

(c) *Post-Collection Aflatoxin Production.*—Amplification of aflatoxin concentration in samples after collection and before analysis needs to be better understood, and additional ways to prevent it should be developed.

The use of volatile fatty acids (e.g., propionic acid) as a preservative should be evaluated both for their ability to stop fungal activity and for the effect of these acids on aflatoxin. Other antifungal compounds should be tested. However, it must be recognized that some such compounds, e.g., formaldehyde and SO_2 gas, also partially degrade aflatoxin and would not be suitable for treatment of samples before analysis. Also, compounds such as propionic and acetic acid are only effective 3–5 months following treatment. The effect of these materials on quantitative analyses for aflatoxin should be investigated. Of paramount importance is the effect of such agents on the containers used to hold the grain.

(d) *Analytical Problems.*—There is an increasing need for an inexpensive, simple, and sensitive multitoxin assay which can be performed on a single extract from a sample.

There is an increasing need for collaborative studies and evaluations on analytical methods for mycotoxins. In particular, the FL-I and FL-IRS methods of Davis and Diener (18, 24) should be evaluated because of their reported sensitivity, reliability, low cost, and use of nontoxic solvents. It would be of great value to have additional presumptive tests for aflatoxin in corn and other grains to supplement the widely used BGYF method, which is the only truly rapid method.

Other Needs

We need to be more careful with the use of the term "survey." Just as random sampling does not mean haphazard sampling, a survey does not mean a collection of assay results from materials of unknown origin and handling. The word survey implies a prior experimental or sampling plan and a *post facto* assemblage of data prescribed by the plan. Proper restriction of the term would contribute much to the development of more meaningful surveys, and provide for better planning.

RECOMMENDED PROTOCOLS FOR SURVEYS, SAMPLING, POST-COLLECTION HANDLING, AND ANALYSIS OF GRAIN SAMPLES RELATIVE TO MYCOTOXIN PROBLEMS

General Considerations

No single protocol can be recommended that will be suitable for every situation involving collection and analysis of grains for mycotoxins. Therefore, the Ad Hoc Work Group recommends protocol guidelines believed to represent best possible practices, in terms of present knowledge. The guidelines must be adapted to suit individual situations as required. These recommendations are intended primarily for use by trained personnel in connection with officially sanctioned surveys, research projects, etc.

It is recognized that the recommended protocol guidelines are somewhat inconvenient and expensive, and require certain equipment and procedures not readily available to non-technical personnel. It is hoped that research recommended elsewhere in this report can eventually provide more convenient and inexpensive procedures. It is important that all personnel receive information regarding necessary safety precautions (not covered in this report) in the handling of solvents, standards for toxins, and preparation of samples.

Protocol Guidelines for Surveys

These guidelines are statistically designed to draw inferences about the frequencies and levels of mycotoxins in grains, particularly aflatoxin in corn, of a defined population:

1. Preliminary Considerations:

(a) The population to be surveyed must be defined as precisely as possible.

(b) Surveys should be uniformly conducted in all areas where information is needed.

(c) Sampling methodology should be uniform so results can be compared.

(d) A suitable sampling frame (to assess farms, fields, bins, etc.) must be identified or developed.

(e) Sampling procedures must be established with proper attention to randomization which is essential so that selectivity and resulting biases can be avoided.

(f) Adequate sample size is required to provide reliable and useful results.

(g) Post-collection mold growth and production of mycotoxins must be prevented.

(h) Adequate comminution of samples is required to uniformly blend any mycotoxin present throughout the sample.

(i) A uniform method of analysis must be selected. Preferably, only methods currently validated and approved for the commodity being surveyed should be used.

(j) A standard format for reporting data should be adopted.

(k) Appropriate procedures for statistical analysis of survey data should be designated.

2. Procedures:

(a) The population must be defined, and the sampling frame to be used must be selected. Selection procedures must be random. In case of field sampling of corn, it would be preferable to harvest an entire field of corn and take representative stream samples of the shelled corn as previously discussed. Alternatively, probe samples can be taken (immediately after combining but before storage). Samples can be collected from randomly selected 1-acre plots, using a table of random numbers to designate plots within the field (25). Field sampling requirements are far more critical if reliable information for the individual field is needed. However, field reliability may not be an objective in some surveys.

(b) Sampling must yield a minimum of one 10 lb sample per unit sampled. Stream sampling is preferable to probe sampling which in turn is preferable to field sampling of ears of corn. However, when the latter is required it is important for ear collection to be carried out without selectivity; if sampling must serve to estimate for an individual field, it must be widely distributed throughout the field. The errors given previously should be considered when choosing the number of ears to be collected for each sample.

The following procedures for randomly selecting the desired number of ears (N) for the sample are suggested for guideline purposes: Diagram each field and divide the field into N approximately equal sections. If the field is rectangular, a count of rows for width and a count of paces for length can be helpful in dividing and identifying N equal field sections. Devise a method to randomly select one ear from each section. Composite the N ears in a mesh bag as a single sample representing the chosen field.

(c) Each sample should be handled in a manner that minimizes the post-collection production of mycotoxins. Moist samples should be held in cloth or paper bags, cooled if feasible, and transported to drying facilities as soon as possible. Samples should not be placed in bags, containers, car trunks, large piles, or other confined situations where humidity and temperature can increase around them. Significant mold growth and toxin production can occur in just a few hours and this must be avoided. As soon as possible after collection, the samples should be dried at approximately 80–90°C for 3 hours or more to reduce the grain moisture to about 12–13% (where molds are to be studied, 60°C for a longer time is recommended). If the samples were refrigerated before drying, they should be dried immediately and kept dry until analyzed.

(d) The entire 10 lb sample should be ground to pass a No. 14 sieve, thoroughly blended, and properly subdivided to a 1 kg sample. The entire 1 kg sample should be ground to pass a No. 20 sieve, thoroughly blended, and properly subdivided to 50 g analytical samples (13). When feasible, the samples may be ground and subsampled immediately after collection. The subsamples can then be analyzed immediately, stored under refrigeration, or dried for storage.

(e) Analysis of all samples for mycotoxins should be done by appropriate methods approved by AOAC (13), AACC (15), AOCS (12), or similar organizations. The method for analysis of the samples must be selected in advance and used uniformly throughout the survey. In the case of aflatoxin in corn, a revised minicolumn method that is currently recommended by AOAC for screening purposes specifies the Holaday extraction and cleanup procedure (19) in conjunction with the Velasco minicolumn (9, 20). Where more precise quantitative data are needed, the AOAC CB method is recommended (13) for determination of aflatoxin in corn.

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Sampling and Preparation of Samples of Peanut Butter for Aflatoxin Analysis

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Procedures are discussed for sampling peanut butter and preparing those samples for aflatoxin analysis. Special emphasis is placed on sampling the product from shipping pallets and comminuting chunk style peanut butter in order to reduce the variability in the analysis associated with the nonuniform distribution of aflatoxin in the product. The slurry method of preparation is a convenient means of obtaining a sample which is representative of a nonhomogeneous product.

The difficulties in obtaining a representative sample of peanuts for aflatoxin analysis have been established in studies carried out by Whitaker *et al.* (1). These difficulties are even more evident when the research performed by Cucullu *et al.* (2) at the Southern Regional Laboratory of the U.S. Department of Agriculture (USDA) is reviewed. These later researchers painstakingly sliced individual peanuts into layers and determined the concentration of aflatoxin in the various segments. This provided profiles of the distribution of aflatoxin within individual nuts. Table 1, which contains data from their published work, clearly demonstrates that the distribution was highly variable. The points of highest concentration were well within the body of the peanuts and surprisingly little could be found where visible mold spores were present. Mathematically, a single peanut containing aflatoxin in the concentration of "Peanut No. 2" would contaminate 100 lb negative nuts to a level of 20 ppb. The probability of detecting the true level of the contamination present therefore obviously depends on the particle size of the product material being sampled.

Three alternative sampling techniques are known to be in use for obtaining samples from chunk style peanut products for aflatoxin analysis:

(1) Averaging the results from multiple samplings, each analyzed by the standard method (3). The work effort with this approach increases proportional to the number of analyses made.

(2) Grinding samples to creamy style consistency with a Straub or Bauer mill and blending for uniformity in a Hobart mixer, followed by analysis by the standard procedure (3) of a single composite sample. The time required to prepare the composite, because of cleaning of the equipment between batches, can overshadow the time saved by the single analysis.

(3) Preparing a slurry and analyzing an aliquot by the standard procedure (3). For the same sample size, this approach requires less time and effort with a slight increase in solvent usage relative to the second approach.

The first two approaches need no further explanation, for they require no unusual sample manipulation. The slurry method, as used by Best Foods, will be detailed in this paper.

Experimental

Weigh 18–54 oz (510.3–1530.9 g) warm chunk style peanut butter into 3 qt blender jar directly on top-loading balance. Samples are most easily transferred if they are relatively warm and fluid soon after processing. However, any peanut butter can be softened in 2–3 min by warming to ca 71°C through submersion of the jar in recently boiled water. Add 200 mL hexane for each 510.3 g peanut butter to explosion-proof blender, and note weight. Because the warm peanut butter causes slight loss of hexane (ca 2–3 mL), weighing should be carried out in fume hood. The loss of hexane is usually too small to be of consequence to the analysis but is automatically corrected for when the hexane addition is also weighed.

Table 1. Aflatoxin B_1 content in constituent parts of selected peanuts[a]

Part	Peanut No. 2, ppb	Peanut No. 16, ppb
Skin	570,000	—
Heart	4,000,000	27,000
Abaxial surface	3,000,000	98,000
Layer 1	2,300,000	—
Layer 2	760,000	—
Mesophyll	650,000	130,000
Adaxial surface	60,000	340,000
Layer 1	—	—
Mold	ND	10,000

[a] Data from ref. 2.

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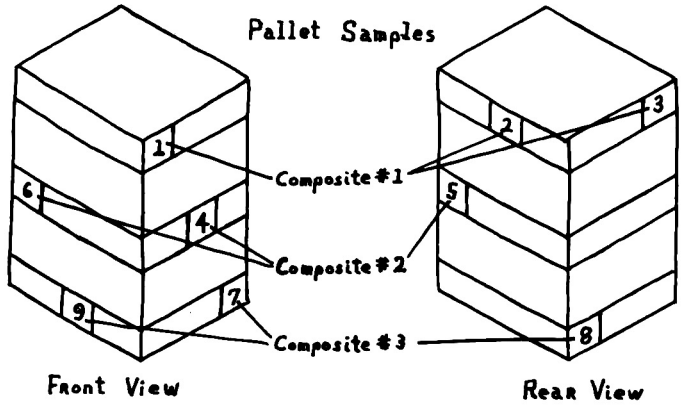

FIG. 1—Pallet sampling for aflatoxin assay.

Stir solution in blender a few times with spatula, cover, and blend 1 min at low speed. Push down product deposited on sides with spatula, and blend 4 min at high speed. Transfer aliquot equivalent to 50 g peanut butter to 1 qt blender jar by weighing directly into jar on top-loading balance. Use AOAC method II (3) with its normal solvent proportions.

Calculate weight of slurry containing 50 g peanut butter as follows:

$$\text{wt of slurry} = (\text{wt of sample} + \text{wt of hexane}) \times \text{wt for assay/wt of sample}$$

$$\text{wt of slurry} = (510\text{ g} + 133\text{ g}) \times 50\text{ g}/510\text{ g}$$

$$\text{slurry used for assay} = 63.0\text{ g}$$

When the previously indicated sample and solvent proportions are used (that is, 510.3 g and 200 mL solvent), the weight should be ca 63 g.

Pallet Sampling

Remove 2 jars from each of the 9 cases specified in Fig. 1. Prepare two 3-jar composites for each of 3 levels of pallet (1 jar from each of the 3 positions on a given level). For creamy style product, only 50 g is required from each jar but for chunk style product, up to 510.3 g is used from each jar.

Results and Discussion

Size distributions obtained by a Coulter Counter of any of the leading creamy style peanut butters on the market indicate that the majority of the particles are less than 8 μm in diameter, and the mean volume is about 11 μm (Fig. 2). Thus a typical florunner peanut with a volume of about 0.8 mL would be divided into well over 300 million particles. This finely divided material becomes widely distributed in the production of creamy style peanut butters. The standard analysis sample of 50 g taken from any jar made over a period of many minutes or even an hour has a high probability of being equal in concentration for any aflatoxin contamination present. The uniformity of the values depends on the volume of the processing line after the grinding of the peanuts. This uniformity, however, is not attained with chunk style product. The latter is a blend of large and small particles where the same typical florunner peanut may be severed into fewer than 50 particles. The impact of this can be demonstrated by blending into chunk style peanut butter chunk pieces from nuts commonly rejected from the processing stream. In one such case, a

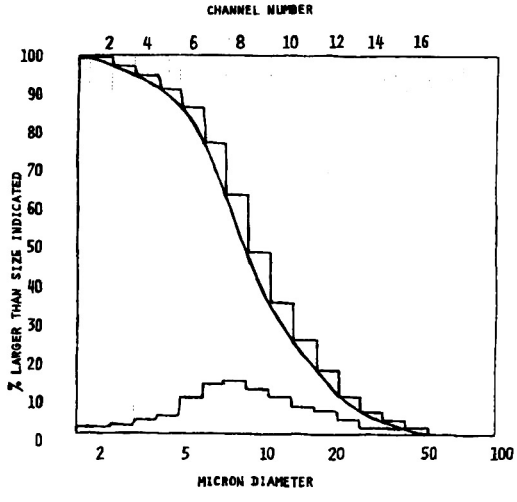

FIG. 2—Typical particle size distribution of creamy peanut butter.

50 g sample contained 135 ppb aflatoxins. Six more analyses from the same jar did not reveal a trace of aflatoxin. Numerous other samples from the same preparation also failed to demonstrate positive values. It clearly demonstrates that an effective sampling program is required if a truly representative value is to be obtained for a chunk style product.

Comparison of creamy style peanut butter assayed by both the slurry method and the standard method (3) confirmed, by the absence of any differences, that preparation of the slurry does not introduce errors in the analysis.

However, the place from which the sample is obtained has a major bearing on minimizing of errors. Before the grinding step in the manufacture of peanut butter, the sampling problems are the same as demonstrated by Whitaker *et al.* (1) for raw peanuts. Following the grinding step, peanut butter processing can take 2 forms: batch process or continuous process. In either case, from the discussion of the effect of particle size on aflatoxin distribution, it can be recognized that the most representative sample should be the one which allows for the greatest amount of mixing to have taken place.

Therefore, the packaged product, or a sample drawn immediately before packaging, has the greatest chance of being representative of the batch or of the product contained at that moment within the equipment of the continuous process. Any sample drawn earlier increases the risk of variability.

Sampling the product after it has left the processing area requires removal of samples from the shipping pallet. Sampling by a government agency has in the past consisted of the removal of individual cases of product which are each reportedly composited into one sample for analysis. By contrast, the sampling program of a processor has been outlined in the experimental section under *Pallet Sampling.*

To demonstrate the relative merit of the 2 sampling techniques, data are presented for experimental chunk style peanut butter prepared from an indemnifiable lot of peanuts. The lot had an average value of 31 ppb. Two 12-jar batches of 12 oz product were prepared and analyzed as individual jars by the slurry method.

The data in Table 2 show 3 jars of the 24 with unusually high values—83, 53, and 87 ppb. All the others were 3 ppb or less. If creamy peanut butter were prepared from the composite of all samples, we would expect to find the indicated 10.9 average for any 50 g sample drawn. If composited as individual batches or cases as they may be, they would have shown 8.7 and 13.1 ppb averages, respectively. Compositing all the jars in one batch appears to give a representative result. However, it is still representative only of that batch and not necessarily of the lot from which it was obtained.

Table 2. Evaluation of slurry method on chunk style peanut butter

	Total aflatoxin content, ppb	
Sample	Batch 1	Batch 2
1	83	53
2	Tr[a]	0
3	3	3
4	3	2
5	3	87
6	2	Tr
7	0	3
8	3	3
9	3	2
10	2	Tr
11	1	2
12	0	0
Batch av.	8.7	13.1
Combined av.	10.9	

[a] Trace.

Any random sampling of the same number of jars spread over a pallet theoretically has a better probability of representing the value of the pallet than drawing only from one case. Figure 3 shows a statistical equation for expressing the error of measurement. This equation for estimating variance has a term for each of the variance components which could contribute error. The numerator is an estimate of variation contributed from the component source and the divisor is the number of samples associated with the component. The square root of the sum of the squares of the components is the standard deviation of the aflatoxin results. A graph of the relationship of the variance components vs. number of samples shows that as the number of measurements increase the contribution to the improvement in error decreases (the difference between 4 and 5 is less than between 1 and 2), and the greater the variability possible (as shown by the 2 curves) the greater the reduction in error by increasing the number of measurements. Earlier it was indicated that extreme variation could occur in the results of different aliquots from the same jar (135 and 0). This variation was eliminated by the total analysis of the jar contents, using the

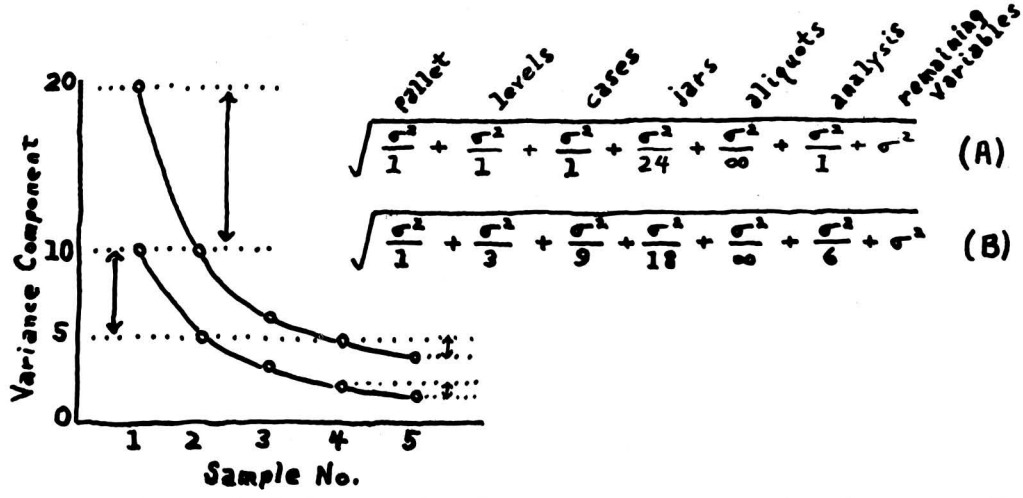

FIG. 3—Statistical and graphical presentation of sampling error: A = 1 composite analysis of 1 case in 1 pallet; B = 6 analyses from 3 composites of each of 2 jars from each of 3 cases on each of 3 levels of 1 pallet.

slurry method, and thus eliminating aliquots from the equation as the denominator went to infinity. Earlier data also showed the possibility of large deviations between jars and smaller deviations among the batches (which for the purpose of this comparison can be considered cases). Thus, the error in aliquots is greater than the error in jars which is greater than the error for cases, etc. If we arbitrarily assign increasing values of 2, 4, 8, 16 to the first 4 variance components, the equations yield the values A = 17.2, B = 12.1. This demonstrates that fewer jars randomly drawn will provide less error in estimation of the true level than will a larger number of samples drawn from a more limited area. Or in other words, random sampling of jars from a pallet is more representative than the analysis of any single case from that pallet.

Acknowledgment

The author thanks R. Leofsky for statistical interpretations.

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Confirmatory Test for the High Pressure Liquid Chromatographic Determination of Aflatoxin B_1

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The iodine derivative of aflatoxin B_1 was determined by reverse phase high pressure liquid chromatography with fluorescence detection. Aqueous solutions of aflatoxin B_1 were chromatographed on a Bondapak C_{18} column with methanol–water before and after treatment with iodine. A 4.5-min shift in retention time and a 25-fold increase in fluorescence resulted from the reaction.

In many laboratories, high pressure liquid chromatography (HPLC) has become the standard method of analysis for aflatoxins, eliminating many of the problems of thin layer chromatography (TLC) (1). Reverse phase HPLC of aflatoxin with fluorescence detection has greatly enhanced sensitivity (2), especially when the aflatoxins are hydrated (3–6). Hydrated aflatoxins have been detected at the subpicogram level by use of HPLC with laser fluorimetry (7). Previously, we reported on the fluorometric analysis of aflatoxin solutions treated with iodine to produce more intensely fluorescing derivatives (8); chromatographic analysis was not involved. The present paper reports use of the iodine derivative as a confirmatory test for aflatoxin B_1 in HPLC analysis.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Waters Model ALC-202 equipped with two M-6000 pumps, 660 solvent programmer, Model U6K injector, and Model 420 fluorescence detector with 360 nm primary filter, 440 nm secondary filter, and F4T5BL lamp (Waters Associates, Inc., Milford, MA 01757).

(b) *Column.*—Waters μBondapak C_{18}. Operating conditions: chart speed, 0.1 in./min; flow rate, 0.7 mL/min; ambient temperature; and 1500 psi pressure.

(c) *Recorder.*—10 mV with chart speed of 10 in./hr (Beckman Instruments).

Reagents

(a) *Solvents.* — Methanol (Mallinckrodt, St. Louis, MO 63147); water, demineralized and glass-distilled.

(b) *Mobile solvent.*—Methanol–water (66+34).

(c) *Aflatoxins B_1, B_2, G_1, and G_2.*—Calbiochem-Behring Corp., La Jolla, CA.

(d) *Aflatoxin B_{2a}.*—Prepare from aflatoxin B_1 by adding HCl (7).

(e) *Saturated iodine solution.*—Add 1 g I_2 to 200 mL water. Stir mixture 15 min and store in the dark in stoppered flask.

(f) *Aflatoxin standard solutions.*—Warm 10 mg aflatoxins (B_1, B_2, G_1, or G_2) in 5 mL chloroform and dilute to 100 mL with benzene. Determine concentration spectrophotometrically after evaporating solvent and dissolving residue in methanol (8). Store vials containing 5-mL aliquots in freezer.

(g) *Aflatoxin aqueous solutions.*—Evaporate appropriate quantities of standard solutions by gently heating under nitrogen. Dissolve residues in 5 mL hot methanol and dilute to 100 mL with water. Further dilute to appropriate concentrations with water for HPLC analysis. Prepare aqueous solutions daily and store away from heat and light.

(h) *Iodine derivatives of aflatoxin.*—Add 1 mL saturated aqueous iodine to 20 mL aqueous aflatoxin solution, then heat 15 sec in boiling water bath. Dilute solutions as required with water. Protect from light and refrigerate if not used immediately for HPLC analyses.

Results and Discussion

Retention times for various aflatoxins are listed in Table 1. Data show that aflatoxin B_1 had a retention time of 12 min, whereas the iodine derivative of B_1 had a retention time of 7.5 min. The mobile phase of methanol–water was slightly modified from that of Stubblefield

Table 1. HPLC retention time of various aflatoxins and aflatoxins derivatives[a]

Aflatoxin	Retention time, min
B_1	12.0
B_2	11.0
G_1	10.0
G_2	9.0
B_{2a}	5.5
I_2-B_1	7.5
I_2-G_1	6.0

[a] Reverse phase C_{18} column, methanol–water (66+34), 0.7 mL/min, 1,500 psi, fluorescence detection.

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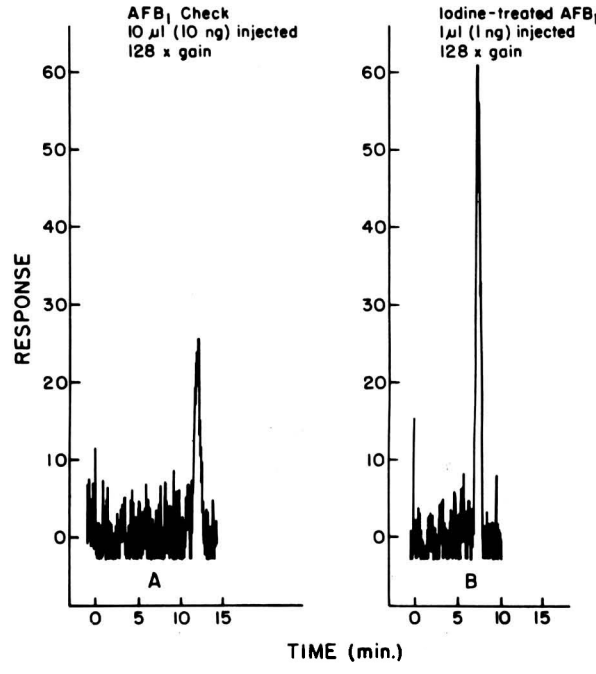

FIG. 1—HPLC chromatograms of an aqueous solution of aflatoxin B_1 (1 ng/µL) before and after treatment with iodine.

and Shotwell (1). The present solvent system did not give baseline resolution of all the aflatoxins when present together in a complex solution. However, the B_1 to I_2-B_1 shift was easily discernible and specific. Similarly, a 10 to 6 min shift in retention time of aflatoxin G_1 after treatment with iodine was a useful confirmatory test for that aflatoxin.

The increase in sensitivity after treatment with iodine is shown in Fig. 1A, as is the shift in retention time. At the maximum gain permitted by the detector, 10 ng aflatoxin B_1 gave a 25% recorder response, whereas, after treatment with iodine, 1 µL treated solution (1 ng equivalent aflatoxin B_1) gave a 60% response. Thus sensitivity was increased about 25 times by treatment with iodine. The shift in retention time from 12 to 7.5 min confirmed the identity of aflatoxin B_1, whereas a similar increase in fluorescence accompanied the shift in retention time in the case of aflatoxin G_1 (data not presented).

HPLC and TLC analyses showed that, although more than one derivative was formed when aqueous solutions of aflatoxins were treated with iodine, the secondary products were minor constituents that did not interfere with the confirmatory test. In our investigation, it was determined that 5 ng aflatoxin B_1 gave a recorder response twice background (128 × gain) and was the lowest level at which aflatoxin B_1 could be determined with absolute confidence. By comparison, 0.2 ng (twice background) of iodine-treated aflatoxin B_1 was easily determined.

Preliminary experiments indicated that the HPLC-iodine treatment procedure can be adapted to various methods of aflatoxin analysis to produce increased sensitivity as well as confirmation of aflatoxin B_1. However, some sample cleanup is necessary or the iodine-aflatoxin treatment may be inhibited. We are preparing a paper on a method for analysis of corn for aflatoxin, using the present HPLC-iodine method. Where necessary, at some point in the analysis, a dry residue can be dissolved in 1–2 mL methanol or DMSO and diluted with water and then treated with iodine solution to give the necessary derivative.

The HPLC method described can be coupled with the FL-I method (8) to give both qualitative confirmation and quantitative analysis for aflatoxins B_1 and G_1 (manuscript in preparation). In such a procedure, it is not necessary to

evaporate samples to dryness in order to prepare the highly fluorescent derivative. Evaporation of samples to dryness is necessary in the production of aflatoxin B_{2a} using HCl or trifluoroacetic acid (7, 9–11). Investigations of the structure and spectral properties of the iodine derivative(s) of aflatoxin B_1 have not been completed. However, investigations to date indicate that, as in the case of aflatoxin B_{2a} (10), fluorescence is much more intense in a water–methanol solution than in absolute methanol, ethanol, chloroform, or hexane. Aqueous systems *per se* also have the advantages of being much less hazardous and expensive than the organic systems customarily used for aflatoxin analysis (8).

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Thin Layer Chromatographic Method for Analysis and Chemical Confirmation of Sterigmatocystin in Cheese

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A semi-quantitative method is described for the analysis of sterigmatocystin in cheese. The method is based on extraction of cheese with MeOH–4% KCl (9+1), followed by Florisil and polyamide column cleanup and 2-dimensional thin layer chromatography (TLC). Visualization of sterigmatocystin on the TLC plates is enhanced by an $AlCl_3$ spray reagent. The identity of sterigmatocystin is confirmed by a 2-dimensional TLC test based on reaction with trifluoroacetic acid (TFA) on the plate after first development. The reaction product formed is visualized by spraying with $AlCl_3$. The method allows detection and confirmation of sterigmatocystin in cheese at concentrations as low as 5 μg/kg. The method has been applied to cheese samples ripening in warehouses and naturally molded with *Aspergillus versicolor*.

Sterigmatocystin is a mold metabolite which has been shown to be toxic and carcinogenic (1–4). The toxin is formed by several species of *Aspergillus* of which *A. versicolor* is the best known. Because *A. versicolor* is a fungal flora found in cheese warehouses and on cheeses (5, 6), it is possible that cheeses intended for human consumption may contain sterigmatocystin. Several studies on the analysis and occurrence of sterigmatocystin in various foods and feeds have been published (7–11), but no analytical procedure has been described for sterigmatocystin determination in cheese. Therefore we developed a method for its analysis and chemical confirmation.

The extraction as well as part of the cleanup procedure of the proposed method are based on the method of Shannon and Shotwell (9). The latter was modified by introduction of an additional polyamide column cleanup step to obtain cleaner extracts. Two-dimensional thin layer chromatography (TLC) is introduced to estimate low sterigmatocystin concentrations. A 2-dimensional TLC confirmatory test is also included in this study.

Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the product by the National Institute of Public Health to the exclusion of others which may also be suitable.

METHOD

Reagents

(a) *Solvents.*—Reagent grade methanol, hexane, methylene chloride, acetone, chloroform, ethyl ether (free of peroxides), ethanol, acetonitrile, benzene, acetic acid.

(b) *Potassium chloride solution.*—Dissolve 4 g KCl in 100 mL water.

(c) *Aluminum chloride solution.*—*See* **26.106**(c) (12).

(d) *Florisil.*—60–100 mesh (Supelco Inc., Bellefonte, PA).

(e) *Polyamide.* — Chromatography grade (Woelm,, Eschwege, GFR).

(f) *Sodium sulfate.*—Anhydrous granular ACS grade.

(g) *Sterigmatocystin standard solution.*—Dissolve sterigmatocystin (Makor Chemicals Ltd, Jerusalem, Israël), to concentration of 5 μg/mL chloroform.

(h) *TLC plates.*—6.7 × 6.7 cm plates cut from 20 × 20 cm plates with 0.25 mm thick layer of silica gel (Merck, DC-Alufolien kieselgel 60, Darmstadt, GFR).

(i) *Trifluoroacetic acid–benzene solution.*—Mix 5 mL trifluoroacetic acid with 20 mL benzene.

Apparatus

(a) *Blender.*—Ultra-Turrax Type TP 18/2 N (Janke & Kunkel KG, Staufen, Breisgau, GFR), or equivalent.

(b) *Filters.*—S&S 595½ folded paper filter (Schleicher & Schüll, Dassel, GFR).

(c) *Chromatographic tubes.*—22 × 300 mm with G2 glass filter and Teflon stopcock; 8 × 100 mm glass tubing without filter and stopcock.

(d) *UV illumination cabinet.*—365 nm. Examine intensity of lamp by illuminating TLC plate under longwave UV lamp at distance of 10 cm. Standard spots containing 10 ng sterigmatocystin should be visible after spraying with $AlCl_3$ solution.

(e) *Syringe.*—50 μL, No. 705 point style 3 (Hamilton Co., Reno, NV 89510).

(f) *Repeating dispenser.*—PB 600-1 (Hamilton Co.).

(g) *Vacuum rotary evaporator.*—Rotavapor RE (Büchi, Apparatefabrik Flawil, Switzerland).

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Extraction

Weigh 20 g grated cheese sample into beaker, and add 180 mL methanol and 20 mL KCl solution. Homogenize 90 sec with Ultra-Turrax. Filter through folded filter paper, transfer 100 mL to round-bottom flask, and concentrate with vacuum rotary evaporator to ca 20 mL. Maintain bath temperature at 38–40°C during evaporation to prevent foaming.

Column Cleanup

Proceed with cleanup method of Shannon and Shotwell (9): Pour ca 75 mL ethanol–water (9+1) into 22 × 300 mm chromatographic glass tube. Add 10 g Florisil to column and stir to form slurry. Let Florisil settle; then drain ethanol–water to ca 5 cm above Florisil. Add cotton wool plug to top of Florisil column and drain solvent to top of plug. Transfer extract to column and drain to top of Florisil at 5–7 mL/min flow rate. Rinse flask with two 10 mL portions of hexane; add hexane rinses to column plus additional 80 mL hexane and elute at 5–7 mL/min. Discard hexane. Elute sterigmatocystin with 200 mL acetone–methylene chloride (5+95).

Dry eluate for 30 min over 25 g sodium sulfate, filter through folded filter paper, and evaporate to dryness with vacuum rotary evaporator. Maintain bath temperature at ca 40°C and transfer residue with three 0.5 mL $CHCl_3$ into 25 mL beaker containing 1 g polyamide powder. Evaporate $CHCl_3$ at 50°C under gentle stream of nitrogen and mix the dry polyamide powder. Place small cotton wool plug in constriction of 8 × 100 mm chromatographic tube and add 0.5 g polyamide. Add extract–polyamide mixture on top of this layer and elute with 80 mL water–CH_3CN (7+3) under diminished pressure, obtaining a flow rate of 5 mL/min. Avoid vapor bubbles. Collect eluate and transfer to separatory funnel. Extract twice with 80 and 30 mL $CHCl_3$, respectively, and evaporate combined $CHCl_3$ extracts to dryness in a vacuum rotary evaporator. Quantitatively transfer residue with $CHCl_3$ into 4 mL glass-stoppered conical tube. Evaporate $CHCl_3$ under nitrogen in heating block at 50°C, and redissolve residue in 100 μL $CHCl_3$.

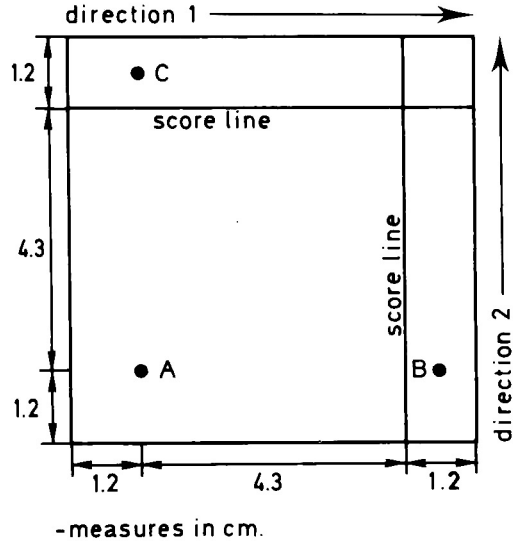

FIG. 1—Schematic of thin layer chromatogram for 2-dimensional chromatography. Direction 1: $CHCl_3$–MeOH (100+2, unlined tank); direction 2: hexane–ethyl ether–HOAc (75+25+10, unlined tank).

A = spotting place for sample extract
B, C = spotting places for sterigmatocystin standards

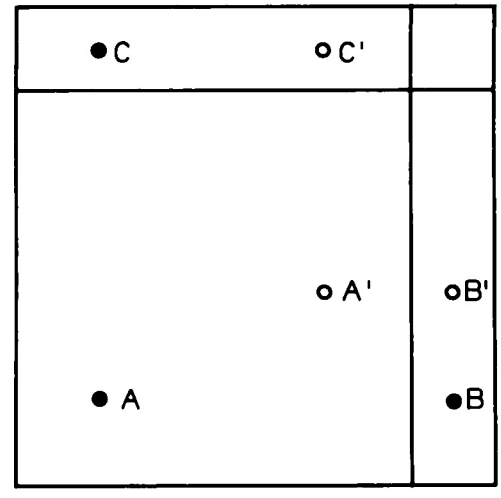

FIG. 2—Schematic of thin layer chromatogram after 2-dimensional development.

A′ = location of sterigmatocystin spot from sample
B′, C′ = locations of sterigmatocystin standard spots

Thin Layer Chromatography

Apply extract and standard spots on 6.7 × 6.7 cm TLC plate according to spotting pattern in Fig. 1. Spot the following aliquots in indicated positions: A, 20 μL sample extract; B and C, 10 μL standard solution (50 ng sterigmatocystin).

Develop plate in first direction with $CHCl_3$–methanol (100+2) in unsaturated tank until solvent front ascends to score line. Air-dry plate for 5 min at room temperature. Develop plate in second direction with hexane–ethyl ether–acetic acid (75+25+10) in unsaturated tank to score line. Dry 15 min at room temperature and spray plate uniformly with $AlCl_3$ solution. Heat plate 10 min at 100°C.

Examine plate under longwave UV light at 365 nm. Spots of sterigmatocystin will give a bright yellow fluorescence. Sterigmatocystin spot from extract can be located at A′ (Fig. 2) with help of

standard spots B′ and C′ originating from B and C. Scan plate with densitometer for semi-quantitative determination (excitation 365 nm, emission 500 nm).

Note: As additional check on location and appearance of sterigmatocystin spot from sample, prepare and develop second TLC plate. Spot at A, 10 μL standard solution (50 ng sterigmatocystin) together with 20 μL sample extract on top of standard spot. Develop as described above and check location of sterigmatocystin by comparison with former TLC plate.

Confirmatory Test

On a new 6.7 × 6.7 cm TLC plate, spot and develop plate in first direction as described above. Dry plate 5 min at room temperature and spray plate uniformly with TFA-benzene (1+4) solution. Let react for 10 min in dark and dry with stream of air for 10 min at 40–50°C. Develop chromatogram in second direction with $CHCl_3$–methanol (100+2). Remove plate from tank when the solvent front reaches score line. Let dry at room temperature for 5–10 min and spray plate uniformly with $AlCl_3$ solution. Heat plate 10 min at 100°C. Examine plate under longwave UV light at 365 nm (Fig. 3). Sterigmatocystin standard from B shows very weak yellow fluorescent spot with a R_f value of unreacted sterigmatocystin (B′) and stronger yellow fluorescent spot of reaction product of sterigmatocystin with TFA at lower R_f (B″). The sterigmatocystin derivative from the extract (A″) has moved the same distance as the sterigmatocystin derivative from B. Identity of sterigmatocystin in extract is confirmed when the R_f values of sterigmatocystin derivative from sample and standard match.

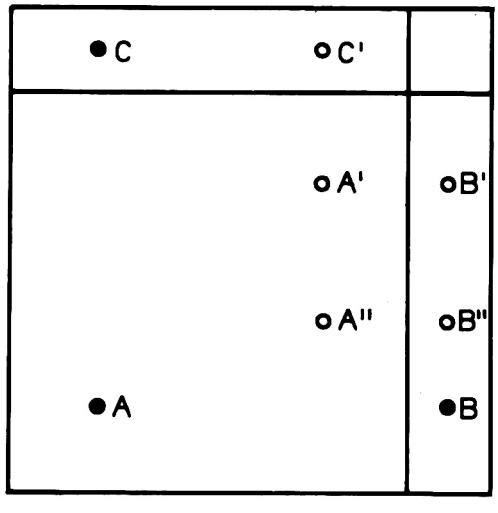

FIG. 3—Schematic of thin layer chromatogram after confirmation.

A′ = residual sterigmatocystin from sample
B′, C′ = residual sterigmatocystin from standards
A″ = reaction product of sterigmatocystin from sample
B″, C″ = reaction products of sterigmatocystin standards

Results and Discussion

The method described above began with the selection of an appropriate extraction solvent for cheese from published methods for analysis of sterigmatocystin in food. Extraction solvents such as acetonitrile-KCl (9 + 1) (8, 10, 11), methanol-KCl (9 + 1) (9), $CHCl_3$-methanol (87 + 13) (7), acetone (Kiermeier, unpublished data), $CHCl_3$, and acetonitrile were tested. The method of Shannon and Shotwell (9) yielded the best recoveries and the cleanest extracts. Fatty final extracts, which hampered concentration and spotting the extract on TLC plates, were obtained when only a Florisil column cleanup was used. Therefore, an additional polyamide cleanup step was included in the method, resulting in much cleaner and less fatty extracts.

A 2-dimensional TLC procedure using 6.7 × 6.7 cm plates was applied because fluorescent background interfered in 1-dimensional TLC analysis of low contaminated samples. The use of small plates not only saves material but also drastically increases efficiency in routine analysis because 10 plates can be developed in one run. For developing, the plates are placed parallel to each other in a stainless steel rack (Fig. 4).

To complete the method, a simple confirmatory test was included in the procedure. Stack *et al.* (10) described a confirmation reaction for sterigmatocystin on a TLC plate based on the reaction between TFA and sterigmatocystin. Because the detection limit of the test did not correspond to the low detection limit of the present analytical procedure, a 2-dimensional confirmation technique was developed, mainly based on the procedure of Verhülsdonk *et al.* (13) for the confirmation of aflatoxins B_1 and G_1.

With our procedure, sterigmatocystin concentrations as low as 5 μg/kg can be detected and confirmed in cheese. This detection limit is low compared to those of existing methods, that vary from 25 to 50 μg/kg (7–11).

The proposed procedure has been successfully applied to the examination of 39 cheese

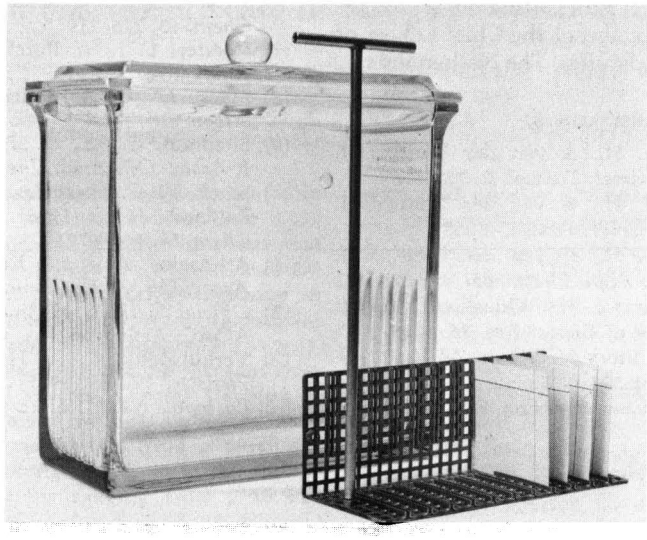

FIG. 4—Stainless steel rack for parallel chromatography of ten 6.7 × 6.7 cm TLC plates.

samples molded with *A. versicolor*; sterigmatocystin was found in the upper 1 cm layer of 9 cheeses at levels of 5–600 μg/kg (6). Fig. 5 illustrates the separation pattern of a cheese extract after 2-dimensional TLC separation. The cheese sample was spiked at a level of 50 μg/kg, which corresponds to 20 ng sterigmatocystin on the TLC plate. Figure 6 shows the same sample following confirmatory analysis.

Recoveries of sterigmatocystin from spiked cheeses with concentrations of 10 μg/kg and 50 μg/kg varied from 30 to 80%.

Acknowledgments

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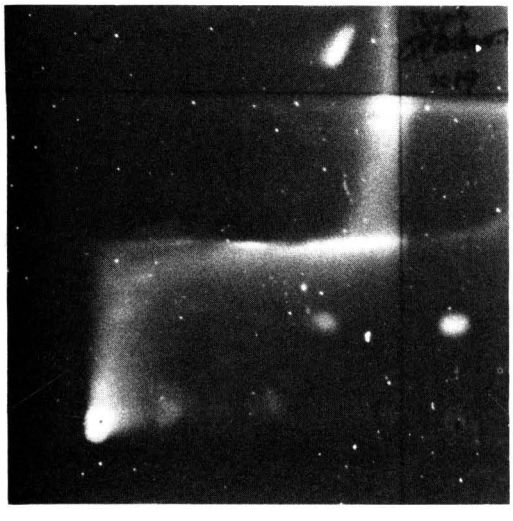

FIG. 5—Two-dimensional separation of cheese extract naturally contaminated with sterigmatocystin (50 μg/kg). For description of spots, see Fig. 2.

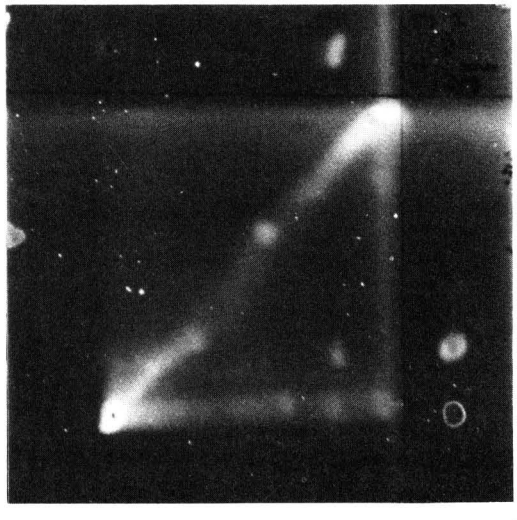

FIG. 6—Two-dimensional confirmation of identity of sterigmatocystin in cheese extract. For description of spots, see Fig. 3.

This investigation was carried out at the request of and for account of the Chief Officer of Public Health (Foodstuffs), The Netherlands.

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REMINDER: Deadlines

Nominations for the 1979 Fellows of the AOAC Award—March 3, 1980

Nominations for the 1979 Harvey W. Wiley Award—April 1, 1980

Nominations for the 1979–1980 Scholarship Award—May 1, 1980

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Fungal Growth and the Presence of Sterigmatocystin in Hard Cheese

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Molds isolated from visibly molded cheeses in shops, households, and warehouses have been identified. Mold flora of cheeses in shops and households consisted mainly of *Penicillium verrucosum* var. *cyclopium*. On cheeses ripening in warehouses, *Penicillium verrucosum* var. *cyclopium*, *Aspergillus versicolor*, *Aspergillus repens*, and *Penicillium verrucosum* var. *verrucosum* were the dominant mold species. Cheeses ripening in warehouses and molded with *A. versicolor* were examined for sterigmatocystin. Nine of 39 cheese samples contained sterigmatocystin in the surface layer in concentrations ranging from 5 to 600 μg/kg.

Cheese can easily become molded during ripening in warehouses and after cutting and slicing during storage in shops or at home. Because mold growth discolors the cheese surface and can affect the flavor, various methods are used to prevent mold growth, such as daily rubbing (1) or treatment with a mold growth inhibiting agent (2, 3). However, where surface flora are not detrimental to the organoleptic quality of the cheese, this laborious treatment is not always carried out with the required frequency. Then, the aerial mycelium of the mold is removed by washing or polishing of the surface of the ripened cheese.

Because certain molds produce toxic metabolites, proliferation of these organisms on cheese must be regarded as a potential health hazard. Also, toxins may penetrate the cheese (4), and polishing its surface might not sufficiently remove them. Thus far, there have been no reports concerning production of mycotoxins in hard cheeses during ripening in commercial warehouses. However, some investigators have suggested that mycotoxins might be found in cheese: Bullerman and Olivigni (5) and Bullerman (6) sampled cheddar and swiss cheeses from shops, and isolated aspergilli and penicillia capable of producing mycotoxins in laboratory media. Bullerman (6) detected penicillic acid in cheese stored for long periods at low temperatures. *Aspergillus versicolor* capable of producing sterigmatocystin has been isolated from hard cheeses (7) but, as far as is known, no cheese has been examined specifically for the presence of sterigmatocystin. Various investigators (4, 8) have found aflatoxins in cheeses after the cheeses were inoculated with *A. flavus*, but there are no indications that this species is common on cheese.

To estimate the occurrence of toxinogenic molds on cheese, molds isolated from cheeses originating from shops, households, and warehouses in The Netherlands were determined according to species. Because of the frequent isolation of *A. versicolor* on molded ripening cheeses, chemical analysis for sterigmatocystin, a carcinogen (9), was carried out in cheeses contaminated with *A. versicolor*.

Experimental

Sampling and Identification of Fungi

Visibly molded cuts of Gouda and Edam cheeses were obtained from shops and households at various places. The cheeses were the bigger types of Gouda (4–15 kg) and Edam (2–4.5 kg). The molds were isolated from the cheeses by streaking onto oxytetracycline yeast extract glucose (OYG) agar plates (10). Visibly molded cheeses of the same Gouda and Edammer types as described above were sampled in 90 warehouses in The Netherlands by streaking the cheese surface with sterile cotton swabs. When a warehouse was visited, each age category was sampled once. More swabs were used when several types of mold occurred. The swabs were streaked onto OYG agar plates. Colonies grown at 24°C for 3–7 days were subcultured on both malt extract agar (Oxoid) and Czapek's solution agar (11). During August 1976–September 1977, all fungal isolates were determined as to species. Aspergilli were identified according to Raper and Fennell (11), penicillia according to Stolk and Samson (12) and Samson *et al.* (13). *Mucorales* were identified according to Zycha *et al.* (14), *Dematiaceous hyphomycetes* according to Ellis (15). Isolates that could not be identified were sent to the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, for identification.

[1] Laboratory for Zoönoses and Food Microbiology.
[2] Laboratory for Chemical Analysis of Foodstuffs.

Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the product by the National Institute of Public Health to the exclusion of others which may also be suitable.

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During October 1977–May 1978, the molds were sampled and isolated as described above, but molds not belonging to the species *A. versicolor* or *P. verrucosum* var. *verrucosum* were not further determined. Warehouses from which *A. versicolor*-positive swabs were obtained were revisited. When the *A. versicolor*-positive cheeses were still present, the cheeses were taken to the laboratory, and the mold, on a marked 25 sq. cm area, was re-examined by streaking onto OYG agar plates. Mold isolates grown on the plates at 24°C were identified. Meanwhile, the cheeses were refrigerated. When the presence of *A. versicolor* was confirmed, the marked square was cut out of the cheese to a depth of 1 cm. Cheese samples were stored for 50–150 days at −18°C until they could be assayed for sterigmatocystin.

A. versicolor cultures isolated from sterigmatocystin-negative samples were checked for their potential to produce sterigmatocystin. They were subcultured on malt extract agar plates and incubated for 14 days at 16°C.

Analysis for Sterigmatocystin

After the plastic coating, which is applied during ripening, was removed, cheese samples of the 1 cm thick upper layer were analyzed according to the method of Van Egmond *et al.* (16). Agar cultures of *A. versicolor* were checked for sterigmatocystin production after extraction with 20 mL chloroform. Chloroform extracts of 20 μL each were spotted on thin layer chromatographic (TLC) plates and developed in hexane–ethyl ether–acetic acid (75+25+10, v/v/v, unsaturated tank). Sterigmatocystin spots were visualized with $AlCl_3$ spray reagent (17).

Effect of Storage on Sterigmatocystin

To demonstrate that cheese samples can be stored at −18°C without loss of toxin, Gouda cheese, aged less than 2 months, was sliced into 1 sq. cm pieces of 0.1 cm thickness. The pieces were inoculated with *A. versicolor* strain RIV 1169 and incubated for 14 days at 16°C. Samples of 5 pieces each were stored at −18°C. On the first day of storage, 5 samples were assayed for sterigmatocystin, whereas 3, 3, and 2 samples were examined after 30, 60, and 90 days of storage, respectively.

Results and Discussion

Tables 1 and 2 reflect the fungal species isolated from molded cheeses sampled in shops and households and from molded cheeses stored in warehouses, respectively. Table 1 shows that cheeses in shops and households were most frequently contaminated with *P. verrucosum* var. *cyclopium*. Because most of the sampled cheeses originated from refrigerated storage facilities in shops and households, it can be concluded that refrigeration does not prevent growth of *P. verrucosum* var. *cyclopium*. *P. verrucosum* var. *cyclopium* is a potential producer of penicillic acid (18), and it seems likely

Table 1. Frequency of incidence of fungal species isolated from molded cheeses sampled in shops and households

Mold species	Shops		Households
	Gouda and Edam cheeses (n = 82)	Processed cheese (n = 39)	Gouda and Edam cheeses (n = 19)
Aspergillus repens	10	1	1
A. versicolor	1		
Cladosporium cladosporioides			1
C. herbarum			1
Fusarium moniliforme	1		
Geotrichum candidum			1
Moniliella suaveolens	1		
Penicillium brevicompactum	1		
P. chrysogenum	3	6	1
P. citrinum	1		
P. verrucosum var. *cyclopium*	65	26	11
P. expansum			1
P. frequentans			1
P. funiculosum	1		
P. verrucosum var. *melanochlorum*		1	1
P. roqueforti	8	5	3
P. verrucosum var. *verrucosum*	7	1	1
Penicillium sp.	2		1
Rhizopus nigricans			2
Scopulariopsis brevicaulis	2	1	

Table 2. Frequency of incidence of fungal species isolated from molded cheeses of different ages sampled in warehouses during 2 periods

Mold species	Age, months <2 (*n* = 36)	2–4 (*n* = 24)	4–7 (*n* = 24)	≥7 (*n* = 8)	Unknown (*n* = 22)	Total (*n* = 114)
August 1976–September 1977						
Aspergillus amstellodami			1			1
A. candidus		4	2	1		7
A. magini		1				1
A. ochraceus				1		1
A. repens	2	8	11	4	3	28
A. restrictus			1			1
A. versicolor	5	3	9	3	4	24
Aureobasidium pullulans	1					1
Cladosporium cladosporioides	1					1
C. sphaerospermum	1					1
Moniliella suaveolens		2				2
Mucor circinelloides					1	1
M. plumbeus			1			1
Penicillium brevicompactum	15	4	1		1	21
P. chrysogenum	3	2	1		5	11
P. verrucosum var. *cyclopium*	19	13	11	4	15	62
P. expansum	1					1
P. funiculosum				1		1
P. verrucosum var. *melanochlorum*	1		2		1	4
P. roqueforti			1		5	6
P. verrucosum var. *verrucosum*	16		1		2	19
Penicillium sp.	2			1	1	4
Scopulariopsis brevicaulis	2		1	1	1	5
S. candida		1				1
S. flava					1	1
S. fusca			2			2
October 1977–May 1978						
	(*n* = 37)	(*n* = 38)	(*n* = 27)	(*n* = 29)	(*n* = 9)	(*n* = 140)
Aspergillus versicolor	11	18	18	24	4	75
Penicillium verrucosum var. *verrucosum*	10	6	2	1	1	20
Other species	19	18	6	7	5	55

that the penicillic acid detected by Bullerman (6) in molded cheeses after storage under refrigeration, was produced by this species. Table 2 demonstrates that *P. verrucosum* var. *cyclopium* was also the predominant species on molded cheeses in warehouses. The following toxinogenic mold species were isolated from warehouse cheese: *A. versicolor*, a well known producer of sterigmatocystin (9), *Penicillium brevicompactum*, which can produce mycophenolic acid (19), and *P. verrucosum* var. *verrucosum*, a producer of ochratoxins (20), citrinin (20), viridicatin (19), and viridicatic acid (19). It is significant that during the second period of investigation of warehouse cheeses, *A. versicolor* was most frequently isolated. During both periods *A. versicolor* was isolated from cheeses from 44 of the 90 warehouses sampled. Furthermore, Table 2 demonstrates that the mold flora of the warehouse cheeses differed with the age of the cheeses: *P. brevicompactum* and *P. verrucosum* var. *verrucosum* were more associated with new cheeses, and *Aspergillus repens* and *A. versicolor* with ripe cheese. This result confirmed the data of Stadhouders and Langeveld (1), who found that the low water activity of ripe cheese favored the growth of some fungal species, including *A. versicolor*. Moreover, they found that this fungal species was able to penetrate into the plastic coating of the cheese. Table 3 summarizes data on the fungicide treatment of the *A. versicolor*-molded cheeses from warehouses. It can be concluded that most sampled cheeses, which were found to be molded, were treated in a way which apparently could not effectively prevent growth of *A. versicolor*.

Table 4 summarizes the results of chemical analyses of ripening cheeses molded with *A. versicolor*. Sterigmatocystin was detected in the

Table 3. *Aspergillus versicolor*-molded cheeses from warehouses distinguished by treatment with fungicides

Period	Treated					Untreated	Unknown
	Period of storage after treatment, weeks						
	0–2	2–4	4–8	≥8	Unknown		
August 1976–September 1977	0	0	0	7	4	5	8
October 1977–May 1978	0	4	9	12	34	5	11

1 cm upper layer of 9 of 39 cheeses. The toxin-positive cheeses originated from 8 warehouses and were found in all categories of cheese age. Concentrations of sterigmatocystin in the test portions ranged from 5 to 600 μg/kg. It should be noted that the test portions were taken selectively from the upper 1 cm layer of the cheeses; therefore, the figures obtained are not representative of the sterigmatocystin content in the whole cheese. All examined *A. versicolor* strains, isolated from cheeses which did not contain sterigmatocystin, demonstrated sterigmatocystin production on malt extract agar. Figure 1 shows that production of sterigmatocystin in cheese inoculated with *A. versicolor* varied widely. It also indicates that sterigmatocystin concentrations in cheeses did not significantly decrease after 3 months of storage at −18°C. This makes it possible to carry out the sterigmatocystin determinations in cheeses some time after sampling.

Conclusions

This is the first published study demonstrating the presence of sterigmatocystin in commercial cheese samples. Sterigmatocystin was detected in parts of ripening cheeses of various ages, which were molded with *A. versicolor*. However, sterigmatocystin may be expected particularly in ripe cheeses, because *A. versicolor* is frequently isolated from ripe cheese. Therefore, from the public health point of view, it is necessary to prevent mold growth from the beginning. From the present results, it can be concluded that, in a number of warehouses, mold growth occurs even after use of fungicides. Because other workers (2, 3) reported that fungicide application could be effective, the reported fungal growth might be due to improper application of fungicides or a lack of hygienic measures, for instance, insufficient disinfection of cheese shelves.

Further investigations are needed to determine the factors leading to fungal growth on cheese.

Table 4. Presence of sterigmatocystin in the upper 1 cm layer of warehouse cheeses molded with *Aspergillus versicolor*

Age of cheeses, months	No. of samples	No. of samples contg sterigmatocystin	Estd concn of sterigmatocystin in upper 1 cm layer, μg/kg
<2	6	1	7
2–4	6	1	50
4–7	8	2	10, 600
≥7	14	3	5, 20, 20
Unknown	5	2	7, 7
Total	39	9	

Acknowledgments

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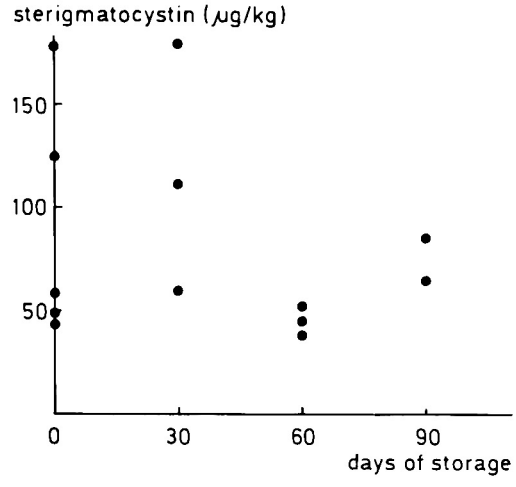

FIG. 1—Sterigmatocystin concentrations in Gouda cheese molded with *Aspergillus versicolor* after different periods of storage at −18°C.

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PESTICIDE FORMULATIONS

Gas-Liquid Chromatographic Method for Determining Bolstar Insecticide in Formulations: Collaborative Study

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A gas-liquid chromatographic (GLC) method for determining Bolstar insecticide in liquid technical material and emulsifiable concentrate formulations was collaboratively studied using Youden's matched pair scheme. Three matched sample pairs were analyzed by 21 laboratories using integrator area measurements and/or peak height measurements. Samples of a technical material containing about 78% active ingredient, a commercial 6 lb/gal. emulsifiable concentrate containing about 64% active ingredient, and a synthetic emulsifiable concentrate containing about 64% active ingredient were dissolved in a measured amount of internal standard solution, tetracosane in toluene, and were subjected to gas-liquid chromatography on a column of 1.5% SE-30 + 1.5% OV-210. Bolstar was detected using flame ionization. The mean coefficient of variation by integrator area measurement for the 6 samples was 1.22%, and the mean coefficient of variation by peak height measurement for the 6 samples was 1.65%. The method was adopted as official first action.

A preliminary study of a gas-liquid chromatographic (GLC) method (1) for determining Bolstar®, which included modification of the manufacturer's method (2) to conform to recommendations of the AOAC Committee on Gas Chromatography (3), was first reported at the 92nd Annual Meeting of the AOAC in 1978. This method and the collaborative data of this paper were also presented at the 23rd Collaborative International Pesticide Analytical Council (CIPAC) meeting, held in Baltimore, MD in June 1979.

Twenty-one collaborators were furnished 6 samples, 3 matched sample pairs, and were requested to perform a single determination on each sample according to Youden's matched pair scheme (4). Samples 1 and 2 were commercial 6 lb/gal. emulsifiable concentrates, Samples 3 and 4 were commercial technical materials, and Samples 5 and 6 were laboratory-synthesized 6 lb/gal. emulsifiable concentrates. The Associate Referee also furnished the 1.5% SE-30 + 1.5% OV-210 column packing, analytical grade Bolstar, tetracosane internal standard, and a standard report form. Collaborators were asked to submit both integrator area results and peak height results along with all chromatograms and integrator print-out sheets. Peak height results alone were requested from the 4 laboratories without electronic integrators.

Bolstar is the trademark of Chemagro, Mobay Chemical Corp., and of Farbenfabriken Bayer Gmb H.

O-Ethyl *O*-[4-(Methylthio)phenyl] *S*-Propyl Phosphorodithioate (Bolstar®) Gas Chromatographic Method

Standard Solutions

(a) *Tetracosane internal std soln.*—Dissolve 2.5 g tetracosane, $CH_3(CH_2)_{22}CH_3$ (Aldrich Chemical Co., No. T875-2) in toluene and dil. to 1 L.

(b) *Bolstar std soln.*—Accurately weigh ca 90 mg anal. grade Bolstar (Mobay Chemical Corp., Chemagro Agricultural Division, PO Box 4913, Kansas City, MO 64120) into 50 mL g-s erlenmyer. Pipet 25 mL tetracosane soln into flask, and swirl to dissolve.

Gas Chromatography

(a) *Gas chromatograph.*—With flame ionization detector, recorder, integrator, and provisions for on-column injection. GLC conditions: temps (°)—inlet 200, column oven 185±5, detector 250; N carrier gas flow (50–75 mL/min) to give retention time of ca 8 min for Bolstar; injection vol. 1.5–2.5 μL; recorder attentuation to give ca 70% full scale deflection for peaks on 1 mV recorder; integrator adjusted to give optimum slope sensitivity, baseline signal, and area response for peaks.

(b) *Column.*—1.2 m (4 ft) × 4 mm (id) Pyrex column packed with 1.5% SE-30/1.5% OV-210 on Gas-Chrom Q. For prepn of column packing, *see* 6.402.

0004-5756/80/6301-0120-08$01.00

Preparation of Sample

Accurately weigh tech. Bolstar or Bolstar emulsifiable conc. contg ca 90 mg pure material into 50 mL g-s erlenmeyer. Pipet 25 mL tetracosane soln into flask, and swirl to dissolve.

Determination

Inject aliquots of std soln until response ratios of Bolstar to tetracosane areas vary $\leq 1\%$ on successive injections. Then make duplicate injections of sample followed by std injection. Calc. av. ratio of Bolstar to tetracosane area for each set of duplicate injections, and calc. % Bolstar.

$$\% \text{ Bolstar} = R \times W' \times P/(R' \times W)$$

Where R and R' = av. integrator area ratios for sample and std, resp.; W = mg tech. material or emulsifiable conc. sample; W' = mg Bolstar anal. std in std soln; and P = purity (%) of Bolstar anal. std.

Results and Discussion

All 21 of the laboratories receiving collaborative samples responded to the Associate Referee's request to perform a single determination on each sample. Seventeen laboratories calculated results from integrator area measurements, and 18 laboratories calculated results from peak height measurements. Collaborators 1, 19, and 21 did not comply with the request for peak height measurements, while Collaborators 10, 15, 16, and 18 did not have access to electronic integrators and could not submit integrator area measurements.

GLC data, using integrator area measurement, from one laboratory could not be used. Investigation revealed that the analyst performed the analyses without having received a letter of instructions. Gross deviation from the collaborative instructions and evidence of GLC temperature control malfunction were reasons for excluding this collaborator's data. Time did not permit repetition of the analyses.

The results for the 20 remaining laboratories were ranked separately for peak height and integrator area measurements according to the amount reported for each sample, with the rank of 1 given to the largest sample. The sum of these rankings for collaborators using integrator area measurements gave no scores outside the 99% probability limits listed by Thompson and Willke (5); however, the sum of these rankings for collaborators using peak height measurements gave only one score (Collaborator 16) outside these 99% probability limits. Consequently, the entire set of peak height data from Collaborator 16 could not be used. Subsequent investigation revealed that Collaborator 16 modified the method by using a flame photometric detector and dilutions.

Two-sample plots (Figs 1, 2, and 3) were constructed for the 3 paired integrator area results

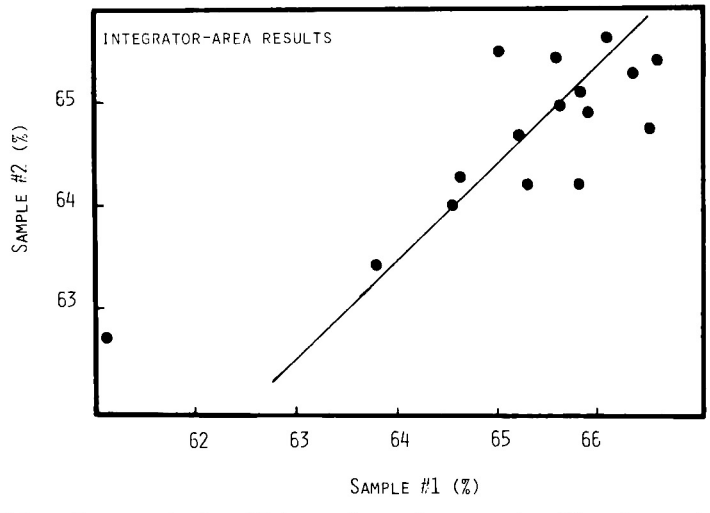

FIG. 1—Two-sample plot of Bolstar pairs, peak area results of Samples 1 and 2.

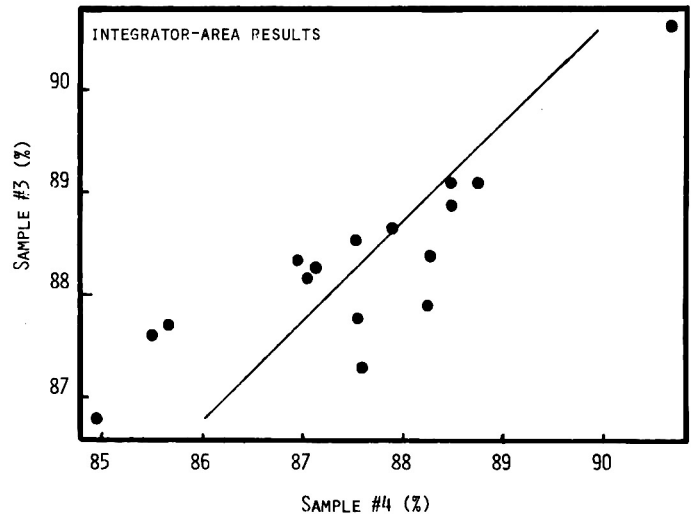

FIG. 2—Two-sample plot of Bolstar pairs, peak area results of Samples 3 and 4.

shown in Tables 1, 2, and 3. Also, 2-sample plots (Figs 4, 5, and 6) were constructed for the 3 paired peak height results shown in Tables 4, 5, and 6. Outliers were visibly identified and were subjected to the Dixon test (6, 7) at the 99% probability level.

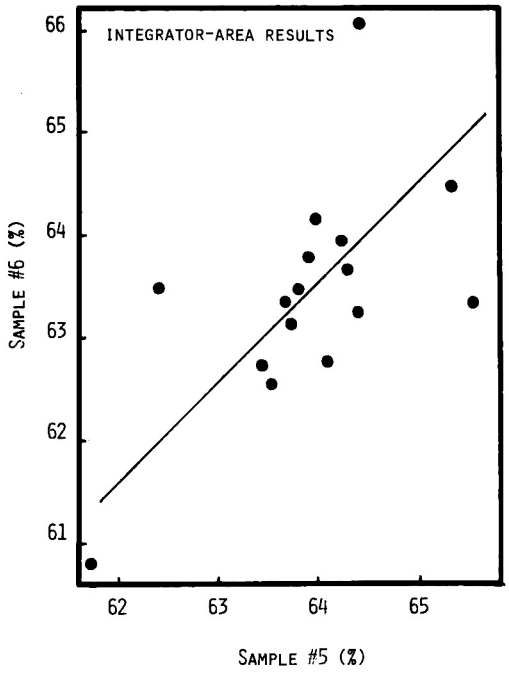

FIG. 3—Two-sample plot of Bolstar pairs, peak area results of Samples 5 and 6.

Consequently, the integrator area results of Collaborator 6 for Sample 1 and Sample 5 were discarded. Also, the peak height results of Collaborator 18 for Sample 3 and Sample 4 were discarded. Investigation revealed extreme ranking scores for both Collaborators 6 and 18, although not outside the Thompson-Willke 99% limits (5). In addition, Collaborator 18 used a high column oven temperature and Collabora-

Table 1. Collaborative results by integrator area for GLC of Bolstar (%) in 64% commercial formulation matched pair samples

Coll.	Sample 1	Sample 2	Diff.	Total
1	65.17	64.74	0.43	129.91
2	66.05	65.66	0.39	131.71
3	64.50	64.04	0.46	128.54
4	65.79	65.16	0.63	130.95
5	65.56	65.00	0.56	130.56
6	61.10[a]	62.73	—	—
7	65.85	64.78	0.87	130.83
8	66.31	65.33	0.98	131.64
9	66.47	64.80	1.67	131.27
11	65.21	64.24	0.97	129.45
12	65.75	64.23	1.52	129.98
13	66.58	65.42	1.16	132.00
14	65.55	65.42	0.13	130.97
17	63.75	63.42	0.33	127.17
19	64.99	65.52	−0.53	130.51
20	64.59	64.29	0.30	128.88
Av.	65.47	64.82	0.66	130.29
S_d	0.79	1.00		0.94
S_r			0.40	
S_b			0.60	

[a] Rejected on basis of Dixon test (6, 7) at the 99% confidence level.

Table 2. Collaborative results by integrator area for GLC of Bolstar (%) in 88% technical matched pair samples

Coll.	Sample 3	Sample 4	Diff.	Total
1	87.27	87.60	−0.33	174.87
2	88.39	88.24	0.15	176.63
3	86.79	84.95	1.84	171.74
4	87.75	87.58	0.17	175.33
5	88.65	87.91	0.74	176.56
6	90.60	90.64	−0.04	181.24
7	88.52	87.54	0.98	176.06
8	89.08	88.48	0.60	177.56
9	88.17	87.07	1.10	175.24
11	88.32	86.96	1.36	175.28
12	87.89	88.22	−0.33	176.11
13	88.22	87.14	1.08	175.36
14	89.09	88.77	0.32	177.86
17	87.71	85.68	2.03	173.39
19	88.87	88.47	0.40	177.34
20	87.59	85.52	2.07	173.11
Av.	88.31	87.55	0.76	175.86
S_d	0.88	1.39		1.55
S_r			0.55	
S_b			1.02	

Table 3. Collaborative results by integrator area for GLC of Bolstar (%) in 64% laboratory synthetic matched pair samples

Coll.	Sample 5	Sample 6	Diff.	Total
1	63.42	62.72	0.70	126.14
2	63.94	63.89	0.05	127.83
3	64.11	62.77	1.34	126.88
4	64.27	63.95	0.32	128.22
5	64.31	63.64	0.67	127.95
6	61.78[a]	60.90	—	—
7	64.40	63.23	1.17	127.63
8	64.45	66.08	−1.63	130.53
9	63.78	63.06	0.72	126.84
11	63.82	63.45	0.37	127.27
12	63.56	62.55	1.01	126.11
13	64.00	64.17	−0.17	128.17
14	65.53	63.33	2.02	128.86
17	62.46	63.48	−1.02	125.94
19	65.36	64.46	0.90	129.82
20	63.70	63.37	0.33	127.07
Av.	64.07	63.61	0.46	127.68
S_d	0.74	0.87		0.94
S_r			0.66	
S_b			0.47	

[a] See footnote, Table 1.

tor 6 reported erratic high and low results, indicative of possible weighing and/or dilution aliquot errors. Otherwise, these 2 collaborators had followed the collaborative instructions, and no further reasons could be found to explain the outlying deviations.

Youden F-test ratios (4), for both the integrator area and peak height data, were calculated to statistically determine the presence of systematic error in the 2 sets of data. For the integrator area data, we found that F-test ratios were greater than the 99% critical values for formulation and technical results, and F-test ratios were less than the 99% critical value for synthetic results. This statistically indicated the presence of bias or systematic error for integrator area formulation and technical results and the absence of bias or systematic error for integrator area synthetic results. For the peak height data, we found that the F-test ratios were less than 99% critical values for formulation, technical, and synthetic results. While this sta-

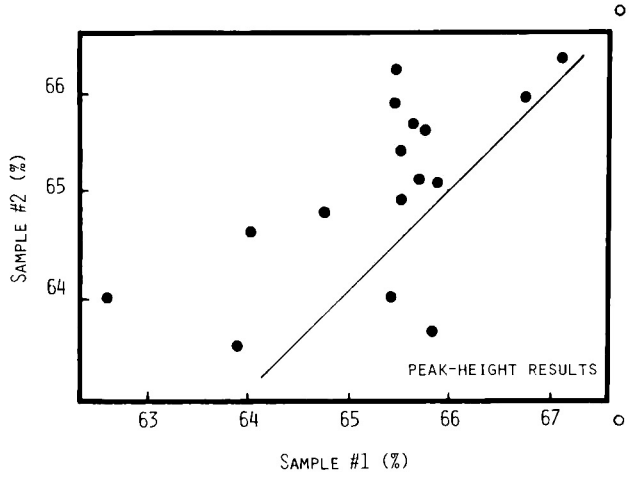

FIG. 4—Two-sample plot of Bolstar pairs, peak height results of Samples 1 and 2.

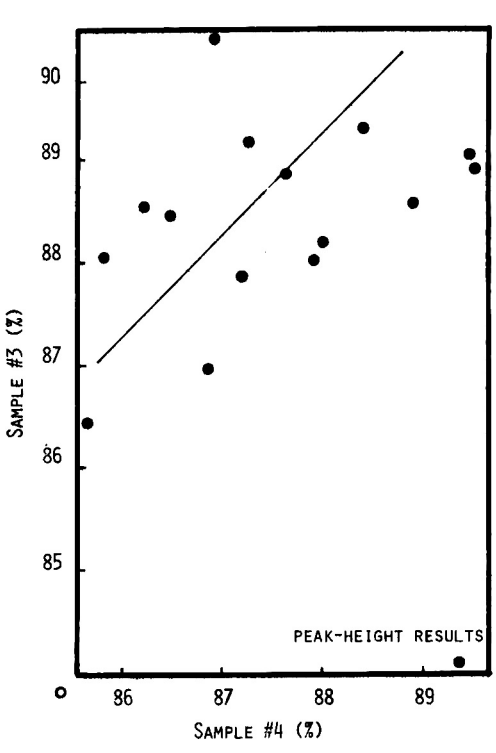

FIG. 5—Two-sample plot of Bolstar pairs, peak height results of Samples 3 and 4.

Table 4. Collaborative results by peak height for GLC of Bolstar (%) in 64% commercial formulation matched pair samples

Coll.	Sample 1	Sample 2	Diff.	Total
2	65.53	64.98	0.55	130.51
3	65.47	65.90	−0.43	131.37
4	65.79	65.67	0.12	131.46
5	64.78	64.84	−0.06	129.62
6	62.61	64.03	−1.42	126.64
7	65.92	65.13	0.79	131.05
8	65.69	65.15	0.54	130.84
9	65.56	65.43	0.13	130.99
10	63.91	63.54	0.37	127.45
11	65.82	63.69	2.13	129.51
12	65.46	64.01	1.45	129.47
13	66.75	65.96	0.79	132.71
14	65.67	65.71	−0.04	131.38
15	67.14	66.32	0.82	133.46
16	70.61[a]	69.19[a]	—	—
17	65.48	66.21	−0.73	131.69
18	69.48	61.57	7.91	131.05
20	64.04	64.63	−0.59	128.67
Av.	65.59	64.88	0.725	130.46
S_d	1.46	1.25		1.24
S_r			1.43	
S_b			—[b]	

[a] Rejected on basis of Thompson and Willke test (5) at the 99% confidence level.
[b] Bias cannot be calculated.

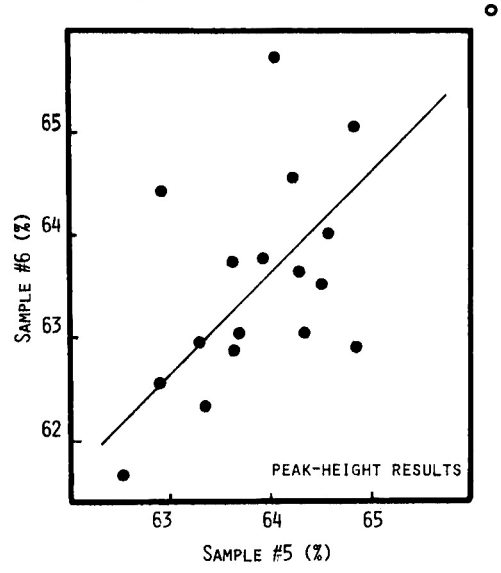

FIG. 6—Two-sample plot of Bolstar pairs, peak height results of Samples 5 and 6.

Table 5. Collaborative results by peak height for GLC of Bolstar (%) in 88% technical matched pair samples

Coll.	Sample 3	Sample 4	Diff.	Total
2	88.20	88.04	0.16	176.24
3	86.95	86.86	0.09	173.81
4	84.01	89.38	−5.37	173.39
5	89.06	89.50	−0.044	178.56
6	90.19	87.00	3.19	177.19
7	89.17	87.33	1.84	176.50
8	88.85	87.69	1.16	176.54
9	88.54	86.28	2.26	174.82
10	88.90	89.58	−0.68	178.48
11	88.45	86.52	1.93	174.97
12	88.01	87.98	0.03	175.99
13	87.84	87.25	0.59	175.09
14	88.58	88.97	−0.39	177.55
15	89.32	88.43	−0.89	177.75
16	93.87[a]	91.34[a]	—	—
17	86.43	85.73	0.70	172.16
18	82.52[b]	81.17[b]	—	—
20	88.05	85.86	2.19	173.91
Av.	88.16	87.65	0.51	175.80
S_d	1.43	1.27		1.34
S_r			1.35	
S_b			—[c]	

[a] Rejected on basis of Thompson and Willke test (5) at the 99% confidence level.
[b] Rejected on basis of Dixon test (6, 7) at the 99% confidence level.
[c] Bias cannot be calculated.

Table 6. Collaborative results by peak height for GLC of Bolstar (%) in 64% laboratory synthetic matched pair samples

Coll.	Sample 5	Sample 6	Diff.	Total
2	63.83	63.66	0.17	127.49
3	64.54	63.53	1.01	128.07
4	64.13	64.56	−0.43	128.69
5	64.84	62.89	1.95	127.73
6	62.57	61.69	0.88	124.26
7	64.36	63.06	1.30	127.42
8	64.06	65.73	−1.67	129.79
9	63.64	62.87	0.77	126.51
10	62.92	64.41	−1.49	127.33
11	63.61	63.76	−0.15	127.37
12	63.37	62.34	1.03	125.71
13	64.60	64.00	0.60	128.60
14	64.30	63.66	0.64	127.96
15	64.84	65.04	−0.20	129.88
16	67.13[a]	67.63[a]	—	—
17	62.90	62.58	0.32	125.48
18	63.30	62.94	0.36	126.24
20	63.72	63.01	0.71	126.73
Av.	63.85	63.57	0.34	127.37
S_d	0.69	1.02		1.04
S_r			0.65	
S_b			0.57	

[a] See footnote, Table 1.

Table 7. Comparison of integrator area and peak height calculations for determination of Bolstar (%)

	Bolstar, %[a]		Coeff. of var., %	
Sample	Peak height	Integrator area	Peak height	Integrator area
1	65.59	65.47	2.23	1.12
2	64.88	64.82	1.93	1.00
3	88.16	88.31	1.62	1.00
4	87.65	87.55	1.45	1.59
5	63.85	64.07[b]	1.08	1.16
6	63.57	63.61[c]	1.60	1.37
Av.			1.65	1.22

[a] Average of results from collaborators.
[b] Theoretical value, 63.86%. Synthetic prepared from technical material (%) technical matched against commercial 98.1% standard.
[c] Theoretical value, 63.09%. Synthetic prepared from technical material (%) technical matched against commercial 98.1% standard.

tistically indicated no evidence for systematic bias, careful observation of each set of peak height result differences reveals that several collaborators, not excluded by the Dixon test, had large magnitudes of result differences. This had the effect of increasing the s_r values to an amount greater than the s_d values with subsequent low F-test ratios.

Table 7 shows excellent agreement of integrator area and peak height results. As would be expected, the precision of the integrator area results is better than the precision of the peak height results. This obvious conclusion is made from the S_r values, precision standard deviations (Tables 1–6), and the average coefficient of variation values (Table 7). The Associate Referee performed, for comparison, 2 sets of t-test (4, 8, 9) calculations, one on Sample 1 (integrator area vs. peak height) averages and the other on Samples 1 and 2 (integrator area vs. peak height) difference of pair totals. The t-values were much less than 99% critical values, and this overwhelmingly indicated no difference in the 2 sets of results. In view of the excellent agreement of the peak height and integrator area averages, the Associate Referee decided not to make further t-test comparison of the 2 calculation methods. Although the peak height method of calculation is acceptable, the integrator area method of calculation is more precise. The use of an electronic integrator for peak area determinations is strongly recommended by the Associate Referee.

The method variables and parameters are tabulated in Table 8. One may draw probable conclusions about extraneous variables and excluded results, because most of the outliers were reported by collaborators who substantially changed the prescribed method parameters. The 21 sets of data strongly indicate that a wide range of variation in method parameters does not affect the results and that the method is sufficiently rugged to accommodate routine variations encountered from laboratory to laboratory.

Recommendation

The Associate Referee recommends that the GLC method for determining Bolstar in formulations be adopted as an official first action method.

Acknowledgments

We wish to thank the following collaborators for their participation in the study:

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This report of the Associate Referee was presented at the 93rd Annual Meeting of the AOAC, Oct. 15–18, 1979, at Washington, DC.

The recommendation of the Associate Referee was approved by the Referee and Subcommittee A and was adopted by the Association. Their reports will appear in *J. Assoc. Off. Anal. Chem.* 63, March issue (1980).

Table 8. Typical variation of collaborator method parameters

		Injection		Column size, mm		Temperature, °C			Carrier gas	
Coll.	Detector[a]	Type[b]	Vol., μL	id	Length	Inlet	Column	Detector	Type[c]	psi
1	F	A	2.5	4	1219.2	240	185	265	N	25
2	F	A	2.6	6.4	1219.2	250	185	250	N	—
3	F	A	3.0	4	1219.2	230	185	210	N	42
4	F	B	2.5	2	1219.2	200	185	250	N	20
5	F	A	2.0	2	1219.2	250	184	250	H	—
6[d]	F	A	3.0	4	1219.2	201	185	250	N	40
7	F	A	2.0	2	1828.8	233	191	247	N	65
8	F	B	1.5	2	1828.8	250	190	250	N	27
9	F	A	1.2	—	1219.2	200	178	230	N	—
10	F	A	2.0	4	1524	200	185	250	N	5
11	F	A	2.6	4	914.4	185	185	250	N	45
12	F	A	0.8	4	1219.2	250	185	300	N	18
13	F	A	2.0	4	1219.2	210	185	260	N	60
14	F	A	1.8–2.0[e]	2	914.4	200	178	250	N	18
15	F	A	1.9	4	1219.2	—	185	250	N	—
16[d]	P	B	2	4	1219.2	260	185	230	N	40
17	F	A	2.5	4	1219.2	200	185	250	N	62
18[d]	F	A	4	4	1219.2	215	200	225	N	—
19	F	A	3	4	1219.2	200	185	250	N	—
20	F	A	2	4	1219.2	205	185	250	N	—
21[d]	—	—	—	—	1828.8	—	215	—	—	—

[a] F = flame ionization detector; P = flame photometric detector.
[b] A = on-column injection; B = separate, glass-lined injector.
[c] N = nitrogen; H = helium.
[d] Collaborator has one or more rejected results.
[e] Variable.

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REMINDER: Deadlines

Nominations for the 1979 Fellows of the AOAC Award—March 3, 1980

Nominations for the 1979 Harvey W. Wiley Award—April 1, 1980

Nominations for the 1979–1980 Scholarship Award—May 1, 1980

For further information and nomination forms for Wiley Award, contact AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209

Gas-Liquid Chromatographic Determination of *N*-Octylbicycloheptene Dicarboximide: Collaborative Study

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A gas-liquid chromatographic method for the quantitative determination of *N*-octylbicycloheptene dicarboximide (MGK® 264 Synergist) was collaboratively studied with technical material and 2 formulations. The samples are diluted with acetone and determined after gas chromatography on an OV-1 column by a flame ionization detector. A statistical evaluation of the matched pair technical samples indicates good agreement among collaborators. An evaluation of the F-factor did not indicate significant systematic error contributions. A statistical evaluation of results for the 2 formulations showed coefficients of variation of 1.25 and 0.97. An examination of the t-values showed no contribution to systematic error. The method has been adopted as official first action.

A preliminary study of the gas-liquid chromatographic (GLC) determination of *N*-octylbicycloheptene dicarboximide (MGK® 264 Synergist, *N*-(2-ethylhexyl)-5-norborene-2,3-dicarboximide) was reported at the 88th annual meeting of the AOAC (1). After subsequent investigation it was decided to retain dibutyl phthalate as an internal standard, and the 5% OV-1 GLC column as opposed to a more polar column. The method given below was used for the collaborative study.

N-Octylbicycloheptene Dicarboximide (MGK® 264 Synergist, *N*-(2-Ethylhexyl)-5-Norborene-2,3-Dicarboximide)

Principle

Sample is dild with acetone contg dibutyl phthalate as internal std. GLC peak ht or area ratios of MGK 264 to dibutyl phthalate peak of sample and std are compared for quantitation. Method is applicable to technical MGK 264 and to several formulations. Not applicable to formulations contg Dursban® and isopropyl palmitate. Presence of large amts of MGK® Repellent 326 causes slightly high results.

This report of the Associate Referee was presented at the 93rd Annual Meeting of the AOAC, Oct. 15–18, 1979, at Washington, DC.

Apparatus and Reagents

(a) *Gas chromatograph.*—With flame ionization detector and 120 cm × 4 mm (id) glass column packed with 5% OV-1 (Analabs, Inc.) on 80–100 mesh Chromosorb W(HP). Operating conditions: temps (°)—column 170, injection port 250, detector 250; gas flows (mL/min)—N carrier gas 60, air 350–400, H 40–50; sensitivity—10^{-10} amp full scale, attenuation 16x. Before use, condition column 2–3 hr at 275° with N flow 50 mL/min. If necessary, vary column temp. or gas flow to attain retention times of ca 6 and 8 min for internal std and MGK 264, resp. Also vary detector sensitivity or injection vol. to attain >100 mm peak ht for each compd (ca 9 μg MGK 264). Theoretical plates/ft must be >300.

Calc. theoretical plates/ft (N) as follows: $N = 16\ L^2/M^2 \times F$), where L = retention GLC peak in mm; M = peak baseline produced by drawing tangents to points of inflection of peak; and F = length of column (ft).

(b) *Internal std soln.*—5.0 mg dibutyl phthalate (Monsanto Co., 98%)/mL acetone.

(c) *MGK 264 std soln.*—Accurately weigh ca 0.15 g MGK (available from McLaughlin Gormley King Co., 8810 Tenth Ave N, Minneapolis, MN 55427) into 50 mL vol. flask, add 10.0 mL internal std soln, and dil. to vol. with acetone.

Determination

Accurately weigh sample contg ca 0.15 g MGK 264 into 50 mL vol. flask, add 10.0 mL internal std soln, and dil. to vol. with acetone.

Inject aliquots (2–3 μL) std soln until ratio of MGK 264 to dibutyl phthalate peak hts (larger peak) or area (use area of both MGK 264 peaks) varies <1% for successive injections. Repeat with sample soln, followed by duplicate injections of std soln. If std peak ratios differ by more than ±1.5% repeat series of injections. Injection vols should not vary more than ±10%. (After elution of MGK 264, it is advantageous to increase column temp. to reduce retention time of subsequent peaks, such as pyrethrins and piperonyl butoxide.) Calc. peak ht or area ratios for duplicate std injections before and after sample injec-

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tions and average the 4 values. Calc. and average peak ht or area ratios for sample injections.

$$\% \text{ MGK } 264 = [(W_s \times P_s)/W_x] \times (R_x/R_s),$$

where W_s = g std; W_x = g sample; P_s = purity of std; R_s = ratio of std; and R_x = ratio of sample.

Collaborative Study

Each collaborator received 4 samples to be analyzed for MGK 264 by the proposed GLC method. Two samples (A and B) were prepared to follow Youden's procedure (2) for closely matched pairs. Samples C and D were MGK intermediates. Also included with the samples was an MGK 264 analytical standard, dibutyl phthalate, and 5% OV-1, the GLC column packing. Of the 15 collaborators, 3 used a 6 ft column, and one used a 3 ft column. Because resolution was adequate, these modifications were considered minor, and the collaborative results were included in the statistical analysis. Four of the collaborators used peak area ratios for calculations. The samples are described in Table 1.

Table 1. Description of collaborative samples for GLC determination of MGK 264 Synergist

Sample	Description	MGK 264 expected, %
A	technical material	92.45
B	technical material	88.80
C	Pyrocide® Intermediate 6030	5.78
D	Pyrocide® Intermediate 6440	18.50

Results and Discussion

Table 2 gives the collaborative results for the 2 samples of technical MGK 264. Using the Dixon criterion (3), 2 outliers were excluded from the original data. Sample A averaged 92.21% (range, 91.22 to 92.90) with a coefficient of variation of 0.42. Sample B averaged 88.66% (range, 88.18 to 89.40) with a coefficient of variation of 0.50. The average difference between A and B was 3.57% as opposed to the expected 3.65%.

From the differences between samples, the standard deviation of random error, S_r, is 0.35, and the overall standard deviation, S_d, is 0.52. The F-ratio (2) of 2.25 meets the minimum of 2.15 required for the presence of systematic errors at the 90% confidence level. At the 95% confidence level, the F-ratio does not meet the minimum required ratio of 2.69. The estimate of standard deviations, S_b, for distribution of systematic errors is 0.27. From the t-value, it can be concluded with 5% risk that the difference between the average per cent found and the expected value is not a result of appreciable systematic error. A graph of a 2-sample chart (of Samples A and B) gives points divided ap-

Table 2. Collaborative results for GLC determination of technical MGK 264 (%)

Coll.	Sample A	Sample B	Diff. (D) A − B	Total (T) A + B	Di-D	Ti-T
1	92.40	89.00	3.40	181.4	−0.17	+0.5
2	92.30	89.40	2.90	181.7	−0.67	+0.8
3	92.13	89.30	2.83	181.4	−0.74	+0.5
4	92.05	88.18	3.87	180.2	+0.30	−0.7
5	92.90	88.79	4.11	181.7	+0.54	+0.8
6	91.30	90.40[a]	—	—	—	—
7	91.86	87.78	4.08	179.6	+0.51	−1.3
8	92.19	89.04	3.15	181.2	−0.42	+0.3
9	92.49	88.43	4.06	180.9	+0.49	0.0
10	92.88	90.43[a]	—	—	—	—
11	92.40	88.50	3.90	180.9	+0.33	0.0
12	91.22	88.39	2.83	179.6	−0.74	−1.3
13	92.38	88.82	3.56	181.2	−0.01	+0.3
14	92.05	88.26	3.79	180.3	+0.22	−0.6
15	92.64	88.67	3.97	181.3	+0.40	+0.6
Av., %	92.21	88.66	3.57	180.9		
Std dev.	0.39	0.44				
Coeff. of var., %	0.42	0.50				
S_d						0.52
S_r					0.35	
S_b						0.27
t-value						1.70

[a] Excluded from statistical evaluation with 95% confidence on the basis of Dixon test (3).

Table 3. Collaborative results for GLC determination of MGK 264 in formulations

Coll.	Sample C[a] Found, g	Sample C[a] Recd, %	Sample D[b] Found, g	Sample D[b] Recd, %
1	5.82	100.7	18.40	99.5
2	5.88	101.7	18.70	101.1
3	5.80	100.3	18.84	101.8
4	5.66	97.9	18.28	98.8
5	5.77	99.8	18.34	99.1
6	5.74	99.3	18.34	99.1
7	5.80	100.3	18.74	101.3
8	5.72	99.0	18.47	99.8
9	5.83	100.9	18.64	100.8
10	5.84	101.0	18.64	100.8
11	5.81	100.5	18.69	101.0
12	5.72	99.0	18.53	100.2
13	5.62	97.2	18.34	99.1
14	5.82	100.7	18.58	100.4
15	5.83	100.9	18.43	99.6
Av.	5.78	100.0	18.53	100.2
Std. dev.	0.072		0.18	
Coeff. of var., %	1.25		0.97	
t-value	0.16		0.65	

[a] MGK® Intermediate 6030 which contains 6.25% technical MGK 264, 6.25% piperonyl butoxide, 5.00% pyrethrins, and 82.50% petroleum distillate.

[b] MGK® Intermediate 6440 which contains 20.00% technical MGK 264, 6.00% pyrethrins, 12.00% piperonyl butoxide, 8.00% petroleum distillate, and 30.00% inerts.

proximately equally among the 4 quadrants, indicating very little systematic error.

The collaborative results for Samples C and D are given in Table 3. The 15 results from Sample C averaged 5.78% (range, 5.72 to 5.88) with a standard deviation of 0.072 and a coefficient of variation of 1.25. The average recovery was 100.0%. Sample D averaged 18.53% (range, 18.28 to 18.84) with a standard deviation of 0.18 and coefficient of variation of 0.97. The average recovery was 100.2%. From the t-values, it can be concluded with 5% risk that the difference between the average per cent found and the expected value is not a result of appreciable systematic error.

The ranking test for laboratories (2) indicates that no one laboratory shows a pronounced systematic error. All collaborator rankings were within the upper and lower limits of 8 and 56, respectively.

Recommendations

The precision of the proposed method as evaluated by this collaborative study is acceptable within AOAC guidelines. The accuracy of the collaborative results agrees well with the expected results. It is recommended that the proposed method for the gas chromatographic determination of *N*-octylbicycloheptene dicarboximide in technical materials and in formulations be adopted as official first action.

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The recommendation of the Associate Referee was approved by the Referee and Subcommittee A and was adopted by the Association. Their reports will appear in *J. Assoc. Off. Anal. Chem.* **63**, March issue (1980).

VITAMINS AND OTHER NUTRIENTS

Application of Gel Permeation Chromatography and Nonaqueous Reverse Phase Chromatography to High Pressure Liquid Chromatographic Determination of Retinyl Palmitate in Fortified Breakfast Cereals

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A high pressure liquid chromatographic (HPLC) method was developed for determining retinyl palmitate in cereals. Retinyl palmitate is fractionated from other methylene chloride-soluble components by using high pressure gel permeation chromatography (HP-GPC) followed by quantitation with nonaqueous reverse phase HPLC (RP-HPLC). HP-GPC fractionation was accomplished using 2 μStyragel (100Å) columns connected in series on sample extracts in methylene chloride with methylene chloride as the mobile phase. A valve designed to facilitate the collection of the vitamin A fraction was installed in-line between the refractive index and absorbance detectors. RP-HPLC quantitation was achieved on μBondapak C_{18} (10 μm) using methylene chloride–acetonitrile (30+70). Based on 23 repetitive analyses, recovery of vitamin A as retinyl palmitate in cereal products was 95.4±4.2% with spiking levels between 21.4 and 140 μg/g. Vitamin A in 15 cereals, representing bran, corn, oats, rice, and wheat, ranged from 34 to 194% of the declared level. The HPLC procedure was compared with the AOAC official colorimetric method and with an ultraviolet spectrophotometric method.

The AOAC official method for the determination of vitamin A in foods and feeds is the Carr-Price colorimetric procedure (1) and in margarine the ultraviolet (UV) spectrophotometric procedure (2). The disadvantages of the antimony trichloride reagent and the associated chromophore have been discussed (3–6). It is generally recognized that a more precise and specific method is needed for the determination of vitamin A active compounds in foods.

Several investigators (7–16) have used high pressure liquid chromatography (HPLC) and each has shown improved precision and specificity. Egberg *et al.* (7) used reverse phase (RP) (Vydac ODS, 10 μm) HPLC to determine all-*trans*- and 13-*cis*-vitamin A as retinol in food products. Dennison and Kirk (8) described a quantitative normal phase (μPorasil) HPLC procedure for determining vitamin A as retinol in cereal products. Head and Gibbs (9) reported on a normal phase (Si-60, 10 μm) HPLC procedure for determining vitamin A as retinol in food composites. Thompson and Maxwell (10) used RP-HPLC (LiChrosorb, 10 μm) to quantitate vitamin A as retinol in margarine, infant formulas, and fortified milk. Cohen and Lapointe (11) incorporated RP-HPLC (μBondapak C_{18}, 10 μm) to determine vitamins A, D, and E in animal feeds. Vitamin A was quantitated as the fortified ester form. The authors concluded that RP-HPLC, with preliminary cleanup, was an efficient technique for analysis of vitamins A, D, and E in animal feeds.

Parris (12) recently reported on the use of nonaqueous RP-HPLC for analysis of low polarity samples such as saturated triglycerides, β-carotene, and fish liver oils containing retinyl palmitate and vitamin D_3. Mobile phases consisted of acetonitrile–methylene chloride mixtures and acetonitrile–tetrahydrofuran mixtures; injection levels up to 10 mg gave no indication of column deterioration during the 8-month study. Holasova and Blattna (13) indicated that gel permeation chromatography (GPC) was suitable for isolation of the ester forms of vitamin A present in fortified foods, thus avoiding saponification which converts vitamin A esters to the less stable alcohol form. Application of small particle (10 μm) supports for high resolution GPC have been reviewed by Vivilecchia *et al.* (14) and Krishen (15).

Recently, Landen and Eitenmiller (16) used a combination of GPC (μStyragel, 100Å), HPLC, and nonaqueous RP-HPLC (μBondapak C_{18}) for the assay of β-carotene and retinyl palmitate in margarine. In the present study, HP-GPC and RP-HPLC techniques were applied to

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the determination of retinyl palmitate in vitamin A fortified dry breakfast cereals. The results were compared with those obtained using the official AOAC procedure (1) and a modified UV spectrophotometric procedure (17).

METHOD

Reagents

See reagents a, b, c, and d in ref. 16.

(a) *Stock standard solution.*—250 μg/mL. Dissolve retinyl palmitate (Tridom Chemical Inc., Hauppauge, NY 11787) in Reagent I.

(b) *RP-HPLC standard solution.*—Transfer 5.0 mL stock standard solution to 50 mL volumetric flask (low actinic), add 10 mL Reagent I, and dilute to volume with acetonitrile. Using mobile phase 2 as diluent, prepare 4 standard solutions containing 1.5, 2.0, 2.5, and 3.0 ng/μL (3.0, 4.0, 5.0, and 6.0 mL standard solution, each diluted to 50 mL). Inject 200 μL aliquot of each and measure peak height and retention volume. Retinyl palmitate response was linear between 300 and 600 ng. Compute ratio response at 313 nm to response at 280 nm.

Apparatus

(a) *Mill.*—Thomas Wiley, Model 4 (Arthur H. Thomas Co., Philadelphia, PA 19105).

(b) *Polytron homogenizer.* — Basic assembly Model PT 10-35 with Model PT 20 ST sawtooth generator (Brinkmann Instruments, Inc., Westbury, NY 11590).

(c) *Liquid chromatograph.*—See ref. 16.

(d) *HP-GPC.* See ref. 16. Continuously monitor eluate at 340 nm for retinyl palmitate fraction with detector sensitivity at 0.10 absorbance unit full scale (AUFS). Use RI at 8X attenuation to monitor oil elution. Set flow rate at 0.7 mL/min (250 psi). For collection, connect specially designed valve in-line between absorbance detector and refractive index (RI) detector. Valve (No. CV-3-HPa, Valco Instrument Co., Inc., Houston, TX 77024) consists of 3-port switching valve with 30° port spacings and make-before-break internal connections.

(e) *RP-HPLC.*—30 cm × 7.8 mm id containing μBondapak C_{18}, 10 μm (Waters Associates, No. 84176) and fitted with pre-column in-line 2 μm filter (Waters Associates, No. 84560), and with guard column, 20 × 3 mm id (Waters Associates, No. 84550) dry-packed (tapping) with μBondapak C_{18}/Corasil (Waters Associates, No. 27248). Continuously monitor eluate at 313 and 280 nm for retinyl palmitate. Set flow rate at 1.0 mL/min (300 psi) and detector sensitivity at 0.05 AUFS for 313 nm and 0.02 AUFS for 280 nm.

(f) *Evaporation system for collected fractions.*—See ref. 16.

Preparation of Sample

Perform all sample preparation procedures under subdued light. Grind dry cereal, using Wiley mill, allowing ground portion to pass 1 mm id stainless steel sieve. For this study, contents of 2 boxes were ground and mixed for composite.

Procedure

For cereals with declared vitamin A levels of 25% Recommended Daily Allowances (U.S. RDA)/oz, weigh 35 g. For cereals with declared vitamin A levels of 100% U.S. RDA, weigh 25 g. Weigh well mixed sample in 250 mL tall form beaker (Pyrex No. 6480), and add 100 mL Reagent I. Place sample in Polytron generator and immerse generator tip at least 4 cm. Homogenize 30 sec at medium speed of 6.

Using vacuum bell jar filtration apparatus, filter through coarse porosity 150 mL fritted glass filter into 250 mL low actinic Erlenmeyer flask. Release vacuum, break up material on filter, add 50 mL Reagent I, mix with spatula, and re-apply vacuum. Repeat wash procedure with 25 mL Reagent I.

For cereals labeled 100% U.S. RDA, add 1 g $MgSO_4$ to combined filtrates and let stand 1 hr (overnight is permissible). For cereals labeled 25% U.S. RDA, use helium to reduce volume of combined filtrates to ca 75 mL with flask immersed in 30°C water bath. Add 1 g $MgSO_4$ and let stand 1 hr (overnight is permissible).

Using vacuum bell jar filtration apparatus and Millipore filter holder, filter filtrate through 0.5 μm fluoropore filter (Millipore, Inc.) into 250 mL Erlenmeyer flask (100% U.S. RDA) or 125 mL Erlenmeyer flask (25% U.S. RDA). For 100% U.S. RDA samples, transfer to 250 mL volumetric flask and dilute to volume with Reagent I. For 25% U.S. RDA level, transfer to 100 mL volumetric flask and dilute to volume with Reagent I.

HP-GPC

Inject 250 μL sample. Follow elution of methylene chloride soluble components with RI and absorbance detectors. Chromatographic pattern produced by both detectors is used as guide for vitamin fraction collection. Major RI detected components eluted in ca 16 min, whereas retinyl palmitate eluted in ca 17 min as indicated by absorbance detector. Initiate collection of vitamin fraction simultaneously with peak response as monitored by absorbance detector and terminate collection when baseline is established. Record maximum digital displayed absorbance value of retinyl palmitate GPC fraction. Collect eluate in 5 mL Reacti-vial, add 10 μL Reagent I (1 μg butylated hydroxyanisole (BHA)), and evaporate just to dryness by attaching vial to closed evaporation system. Operate evaporation system with helium flow at 180 mL/min and reduced pressure

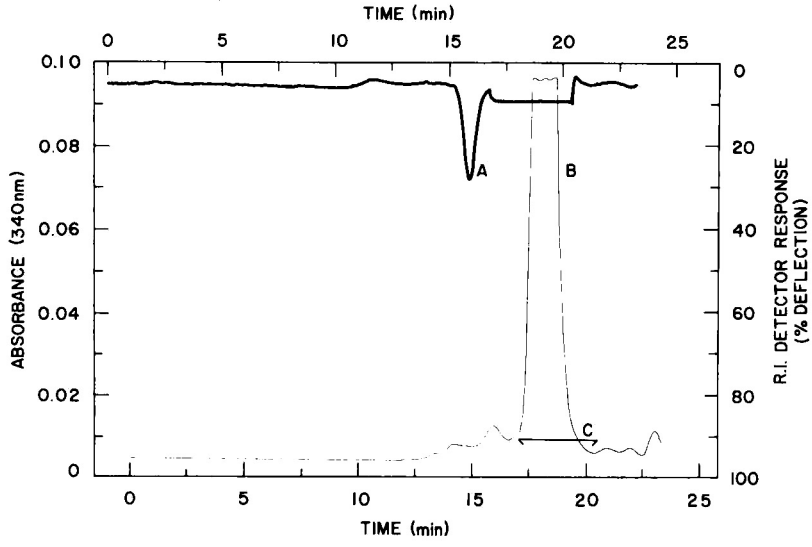

FIG. 1—Chromatogram of vitamin A-fortified low fat cereal on μStyragel, using methylene chloride containing 0.001% triethylamine at a flow rate of 0.7 mL/min. Vitamin fraction was monitored at 340 nm and lipid fraction was monitored by RI. Peak A, lipid; B, vitamin fraction; C, collected region.

(15–20 in. Hg) with vial immersed in 20°C water bath.

RP-HPLC

Use recorded maximum absorbance value for GPC collected fraction to estimate retinyl palmitate levels. Dilute with mobile phase 2 (normally 1.0–2.0 mL) to give concentration between 1.5 and 3.0 μg/mL. Inject 200 μL with flow rate at 1 mL/min and detectors at 313 and 280 nm for retinyl palmitate. Identify compounds from peak retention time and compare response to standard injections at 313 nm. Compute ratio of response at 313 nm to response at 280 nm and compare to standard response ratio.

Results and Discussion

Elution characteristics of retinyl palmitate, retinyl acetate, and retinol were previously established on μStyragel (16). Figure 1 shows the resolution of RI detected methylene chloride-soluble components from the UV-absorbing components during one pass through μStyragel on a vitamin A-fortified rice base cereal (25% U.S. RDA/serving) labeled to contain no fat. Figure 2 shows the same separation on a vitamin A-fortified wheat base cereal (25% U.S. RDA/serving) labeled to contain 1 g fat/1 oz serving. The greater RI response of the lipid fraction is resolved from the collected fraction. The chromatographic pattern produced by the RI and absorbance detectors was used as a guide for vitamin fraction GPC collection. The peak pattern varied from product to product depending on the cereal base and the presence of other components. Since vitamin A content in the different cereals assayed ranged from 34 to 194% of the declared value, the maximum absorbance value of the retinyl palmitate GPC fraction was used to estimate vitamin A concentration in the collected fraction. Therefore, proper dilution was determined to give concentrations between 1.5 and 3.0 μg/mL.

The vitamin fraction was collected manually using the Valco collection valve installed in-line between the absorbance detector and the RI detector on the HP-GPC system. The major RI detector components eluted in about 16 min, with retinyl palmitate elution detected in about 17 min as indicated by the absorbance detector. Collection was started when the absorbance detector responded and ended when a baseline was established. Recovery studies using different breakfast cereals containing added retinyl palmitate validated the collection technique.

Figure 3 shows the RP-HPLC chromatogram of standard retinyl palmitate on dual absorbance detectors at 313 and 280 nm. A peak response ratio (313/280) was used as an indicator of peak purity. The mean peak response ratio established under the specified instrument parameters on 38 standard retinyl palmitate injections was 1.56±0.01. BHA and vitamin E,

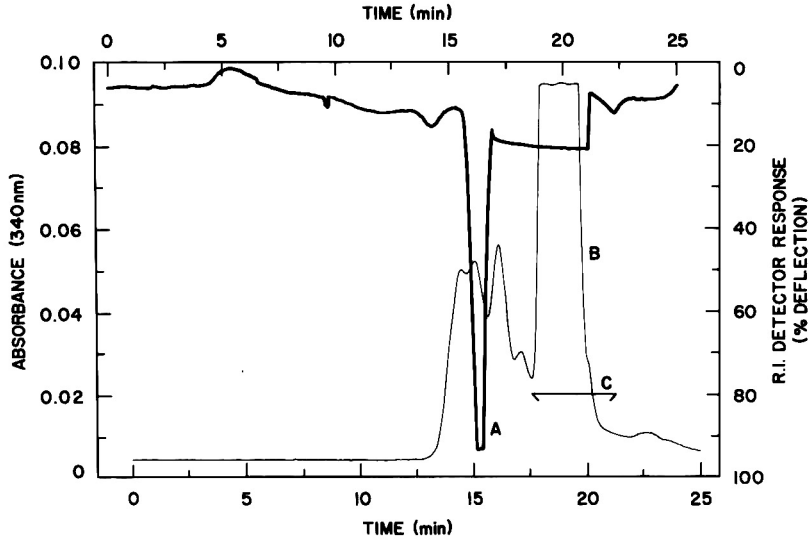

FIG. 2—Chromatogram of vitamin A fortified cereal (1 g fat/oz) on μStyragel. Chromatography parameters were identical to those noted in Fig. 1. Peak A, lipid; B, vitamin fraction; C, collected region.

α-tocopheryl acetate (not shown), were detected at 280 nm and eluted at approximately 11 and 19 min, respectively, by the RP-HPLC system.

Figure 4 is an RP-HPLC chromatogram of a rice base cereal fortified with vitamin A, after one pass through μStyragel, at 280 and 313 nm.

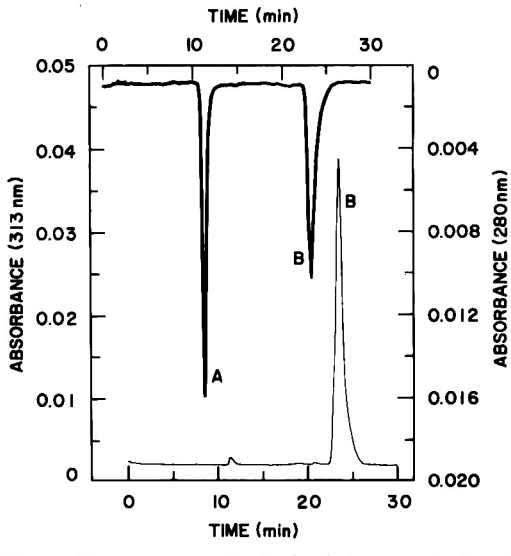

FIG. 3—Chromatogram of retinyl palmitate standard and BHA on C_{18} reverse phase column, using methylene chloride–acetonitrile (30+70) at flow rate of 1.0 mL/min. Response was monitored at 313 and 280 nm. Peak A, BHA; B, retinyl palmitate (500 ng).

Figure 5 is an RP-HPLC chromatogram of a wheat base cereal fortified with vitamins A and E, after one pass through μStyragel, at 280 and 313 nm.

Application of the method to quantitation of retinyl palmitate in duplicate portions of 15 fortified breakfast cereals, with vitamin A label declarations of 25 and 100% U.S. RDA, revealed that retinyl palmitate was the only detectable form of vitamin A. Each composite was prepared from a different lot except for Samples 1 and 8. In Table 1 HPLC results are compared with those from the AOAC procedure (1) on 7 samples and with the UV procedure (17) on 11 samples. The HPLC procedure, using Polytron homogenization in methylene chloride for extraction, tended to give higher results. The HPLC peak response ratio (313/280) for the samples was 1.56±0.01, indicating peak purity as established by the standard peak response.

The UV method (17) uses the absorbance ratio of 0.73 at 300/325 nm to establish spectrum purity for some products. None of our sample absorbance ratios met this requirement. Distortion of the spectrum below 325 nm varied from sample to sample and may be due to the presence of BHA, butylated hydroxytoluene, vitamin E, or other unknown factors. All sample UV spectra showed a plateau in absorbance at 325 nm; these absorbance values were used to calculate the vitamin A level for the UV method as recorded in Table 1.

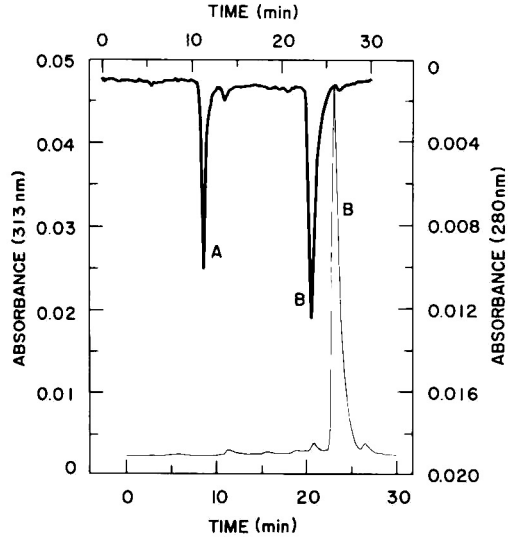

FIG. 4—Chromatogram of vitamin fraction from rice base cereal fortified with vitamin A after one pass through μStyragel on C_{18} reverse phase column. Chromatography parameters were identical to those noted in Fig. 3. Peak A, BHA; B, retinyl palmitate.

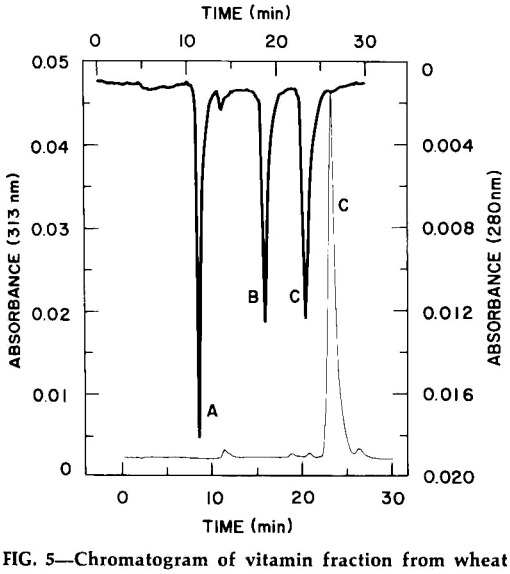

FIG. 5—Chromatogram of vitamin fraction from wheat base cereal fortified with vitamins A and E after one pass through μStyragel on C_{18} reverse phase column. Chromatography parameters were identical to those noted in Fig. 3. Peak A, BHA; B, α-tocopheryl acetate; C, retinyl palmitate.

Other breakfast cereals, not commercially fortified with vitamin A, gave no RP-HPLC interference with retinyl palmitate at 280 or 313 nm, based on normal sample weight injections. Cereal product bases represented by these samples included wheat bran, milled rice, whole wheat, and corn. The products were used in 23 recovery experiments with standard retinyl palmitate added after each composite portion was weighed. The spiking levels ranged from 21.4 to 140 μg/g, with 1 level at 140, 6 at 100, 6 at 35.7, 9 at 28.6, and 1 at 21.4 μg/g. The mean recovery of retinyl palmitate was 95.4±4.2%.

The HP-GPC system, in combination with nonaqueous RP-HPLC, appears to provide a sound technique for the quantitation of vitamin A as retinyl palmitate in fortified ready-to-eat breakfast cereals. The advantages of the procedure include quantitation of the more stable vitamin ester form; nondestructive procedure requiring no heat; and continuous GPC eluate absorbance intensity monitoring that enables estimation of retinyl palmitate levels before the determinative step.

Table 1. Determination of retinyl palmitate (μg/g) in fortified breakfast cereals[a]

Sample	Cereal base	% U.S. RDA fort. level	HPLC	UV[b]	AOAC[b]
1	rice	25[c]	31.3	26.0	26.2
			30.6	26.9	25.4
2	wheat	100[d]	58.7	66.0	65.2
			59.0	69.0	66.5
3	rice	25	33.3	29.1	21.8
			33.7	28.8	22.8
4	corn	25	31.2	24.6	25.2
			31.7	22.1	25.5
5	wheat	25	19.6	20.8	22.4
			18.3	19.2	21.8
6	oat	25	8.2	7.4	7.6
			8.3	7.3	7.4
7	corn	100	91.5	87.5	84.6
			89.7	89.8	84.3
8	rice	25	33.9	30.8	ND[e]
			34.9	30.9	
9	wheat	25	16.0	16.4	ND
			15.8	16.6	
10	corn	100	190.0	174.0	ND
			186.0	174.0	
11	wheat	100	72.8	66.6	ND
			69.1	66.7	
12	rice	25	25.0	ND	ND
			24.7		
13	corn	25	30.0	ND	ND
			30.5		
14	bran	25	46.5	ND	ND
			42.2		
15	bran	25	34.0	ND	ND
			34.6		

[a] Duplicate determinations.
[b] Determined as retinol on saponified sample.
[c] 25% U.S. RDA/oz = 24.25 μg/g as retinyl palmitate.
[d] 100% U.S. RDA/oz = 97.0 μg/g as retinyl palmitate.
[e] ND = not determined.

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PRESERVATIVES

Liquid Chromatography with Amperometric Detection for Determining Phenolic Preservatives

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Liquid chromatography with amperometric detection is a rapid and sensitive method for determining commonly encountered phenolic antioxidants including 3(2)-*tert*-butyl-4-hydroxyanisole, *tert*-butylhydroquinone, *n*-propyl gallate, nordihydroguaiaretic acid, and 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), and the antimicrobial parabens, in a variety of commercial products. Sample extracts are chromatographed directly with few interferences on the reverse phase system. The typical linear range extends from 10^{-11} to 10^{-6} mole of injected analyte. Pertinent experimental factors are discussed with regard to the requirements of the detector and optimizing the determination of this class of compounds.

Many of the chemicals used to protect foods, pharmaceuticals, and cosmetics from damage by oxidation and microbial attack are phenols. Phenolic antioxidants, often in combinations of 2 or more, are used in products containing fats and oils that are susceptible to rancidity. Some major antioxidants are 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), *n*-propyl gallate (PG), and nordihydroguaiaretic acid (NDGA). Other phenols, most notably the homologous esters of 4-hydroxybenzoic acid, the parabens, inhibit the growth of microorganisms. The esters commonly used are methyl-, propyl-, and butyl-*p*-hydroxybenzoate, MPHB, PPHB, and BPHB, respectively. Ethyl paraben (EPHB) usually serves as an internal standard for determinations. The use of these compounds has increased as the use of other agents such as hexachlorophene has become controversial. Concern for the safety of products potentially contaminated with bacteria, particularly cosmetics used near the eye, has also stimulated widespread use of phenolic additives.

A variety of analytical methods have been applied to these phenols. Recent developments in the determination of food additives have been reviewed (1). Thin layer and gas chromatographic procedures predominate. In recent years, liquid chromatography has been applied to the determination of preservatives in foods (2), pharmaceuticals (3), and cosmetics (4). Its major advantages over gas chromatography are the straightforward isolation procedures and lack of a derivatization step. Electrochemical methods such as linear sweep voltammetry (5) and differential pulse polarography (6) have been used for estimating antioxidants in oils and some foods. These methods are rapid and sensitive. Unfortunately the voltammetric oxidation waves for many compounds overlap, severely limiting the usefulness of electrochemical techniques. Liquid chromatography with amperometric detection can provide significant improvements by combining the resolution of a chromatographic system with the sensitivity of electrochemical measurements (7). For example, ion exchange chromatography with amperometric detection has been applied to the determination of ascorbic acid (8) and uric acid (9), and diethylstilbestrol (10) in foodstuffs. We applied liquid chromatography with amperometric detection to the determination of phenolic preservatives, and describe here some factors affecting the performance of amperometric detectors for this class of analyte.

METHOD

Apparatus

(a) *Liquid chromatograph with electrochemical detector.*—Model LC-154 (Bioanalytical Systems, Inc., West Lafayette, IN 47906) with Model LC-4 amperometric detector with additional detector cells made of Teflon, and Kel-F Models TL-4 and TL-5, with carbon paste (CP-O) and glassy carbon working electrodes, respectively. For analyses with a mobile phase containing greater than 30–50% nonaqueous solvent (alcohol or acetonitrile), the glassy carbon working electrode with the opposing

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counter electrode TL-5A is preferred. Loop valve injector (Rheodyne, Berkeley, CA 94710) with 20 μL injection loop and 15 cm × 4.2 mm stainless steel column packed with μBondapak® C_{18}, 10 μm reverse phase material (Waters Associates, Inc., Milford, MA 01757). Flow rate 1 mL/min. Recorder Model RYT (Bioanalytical Systems) with 1 V full scale input range.

(b) *Ultraviolet (UV) detector.*—Model SF 770 Spectroflow (Schoeffel Instrument Corp., Westwood, NJ 07675). See Table 1 for wavelength settings. Use 10 mV recorder range.

(c) *Cyclic voltammograph.*—Model CV-5 (Bioanalytical Systems). Potentials are measured vs. Ag/AgCl/3*M* NaCl reference electrode at room temperature. Scan rates are 200 mV/sec on ca $10^{-5}M$ solution of phenols in mobile phase below. Not essential for routine application of present method, but useful in developmental work.

Reagents

(a) *Salts for buffers.*—Sodium phosphate dibasic, anhydrous, ACS grade (Fisher Scientific Co., Fair Lawn, NJ 07410); sodium phosphate monobasic, ACS grade; glacial acetic acid, analytical reagent grade; ammonium hydroxide, analytical reagent grade (Mallinckrodt, Inc., St. Louis, MO 63147).

(b) *Phenol standards.*—MPHB, EPHB, PPHB, BPHB (ICN Pharmaceuticals, Cleveland, OH 44128); BHA (Sigma Chemical Co., St. Louis, MO 63178); Ionox-100 (2,6-di-*tert*-butyl-4-hydroxymethylphenol) (Pfalz and Bauer, Stamford, CT 06902); NDGA, PG, BHT, TBHQ (97%) (Aldrich Chemical Co., Inc., Milwaukee, WI 53233). Prepare 10-200 ng/20 μL nitrogen-saturated 50% methanol solutions for injection, and store refrigerated.

Table 1. Chromatographic, electrochemical, and ultraviolet absorbance properties of selected phenols

Compound	k′[a]	$E_{p,a}$[b]	Detector potential,[c]	λ_{max},[d] nm
Antioxidants				
PG	0.35	0.41	0.70	275
TBHQ	0.82	0.45	0.80	293
BHA	2.6	0.67	0.80	291
NDGA	3.4	0.37	0.70	287
Ionox-100	5.9	>1.0	1.10	278
BHT	>15	—	—	278
Parabens	—[e]	0.97	1.10	252

[a] Mobile phase: 0.01*M* phosphate buffer (pH 5) in 55% methanol.

[b] Anodic peak potentials obtained from voltammograms of $10^{-5}M$ solutions in mobile phase, using carbon paste.

[c] Working electrode: either carbon paste or glassy carbon (TL-4 and TL-5, respectively) with 5 mil gasket. Reference electrode Ag/AgCl, 3*M* NaCl.

[d] Ref. 11 and experimentally measured using spectra obtained on a Cary-14 scanning spectrophotometer, methanol as solvent.

[e] k′ for individual esters, methyl 0.63, ethyl 1.1, propyl 2.0, butyl 3.7.

(c) *Solvents.*—Deionize and distill all water. Use distilled methanol (spectrograde, or equivalent) low in metal ion contamination. Use good grade of petroleum ether and ethanol which gives no spurious response for blank samples.

(d) *Mobile phase.*—Use mobile phases containing either phosphate or ammonium acetate buffer as supporting electrolyte. pH range (5–6) reduces chance of on-column oxidation of more labile antioxidants, PG, TBHQ, and NDGA. Oxidation potentials shift to higher values with lowering of pH. Proportions of methanol required for acceptable elution will vary with many factors such as column age and condition and analyte. Methanol-water (1+1, v/v) was used for most of development and sampling.

Prepare and store aqueous buffers at 1*M* levels to minimize microbial growth. Prepare 0.5 L batches of mobile phase by filtering sufficient buffer and water (to give final molarity desired) through 47 mm 0.22 μm membrane filter (Millipore Corp., Bedford, MA 01730). Combine with needed volume of freshly distilled methanol in well cleaned and rinsed round-bottom flask with magnetic stirring bar. Draw aspirator vacuum on solution warmed by heat of mixing to deaerate while stirring 2-3 min. Phosphate and ammonium acetate buffers gave similar results.

Extraction Procedure

Antioxidants in oils.—Weigh sufficient sample (ca 0.1–1.0 g) into 20 mL screw-cap culture tube to give concentration of 10–200 ng antioxidant/20 μL (0.5–10 μg/mL) in final extract volume. Dissolve oil in 5 mL petroleum ether. Add solution for standard addition containing 150 μg antioxidant. Extract solution with three 5 mL portions of 80% ethanol. Shake tubes 3–5 min, centrifuge 2 min at 1000 rpm and drain lower layer. Combine extracts in 50 mL volumetric flask and dilute to volume with 50% aqueous methanol.

Antioxidants in foods.—Extraction procedure for oils was applied to dry foods. Grind sample to fine powder with mortar and pestle. Weigh ca 1 g sample into 20 mL culture tube. Add 100 μL standard addition and extract solid directly with 50% methanol in manner described above. Filter final diluted extracts if particulate material is present. Use 5 mL plastic syringe (Fisher Scientific Co., Pittsburgh, PA 15219) to force sufficient volume through Swinnex filter (Millipore) with 0.22 μm filter.

p-Hydroxybenzoates in cosmetic emulsions and suspensions.—Preparations containing benzoates were treated in manner similar to that described by Fitzpatrick *et al.* (4). Weigh 0.1–0.3 g sample

into 50 mL volumetric flask. Add internal standard solution containing ca 1 mg EPHB/mL methanol. Add 10 mL methanol. Extract by shaking and warming flask to ca 60°C for 5–10 min. Cool to room temperature, and then dilute solution to volume with 50% methanol. Centrifuge and filter a small portion, as above, before injection.

Results and Discussion

Voltammetry

An initial study of the electrochemical properties of the selected phenols was carried out by cyclic voltammetry. The results are summarized in Table 1, with chromatographic and ultraviolet absorbance data. Voltammetry readily provides information for ascertaining an appropriate electrochemical detector operating potential. A simple guide for an initial choice is to operate 50–100 mV more positive than the $E_{p,a}$ (anodic peak potential) determined for an individual compound.

The data indicate a distribution of $E_{p,a}$ values for individual antioxidants throughout the range available to the detector. NDGA and TBHQ are detectable even at low potentials, 0.5 V. Most of the antioxidants except BHT and Ionox-100 would give a response for potentials of 0.7–0.8 V. The parabens and Ionox-100 require 1.0 V or greater. The oxidation current of BHT (if it did occur) could not be discerned from the currents due to supporting electrolyte oxidation.

Detector Performance

Because the column effluent must also serve as the medium for electrochemical reactions in the detector, the mobile phase containing large proportions of organic modifier must be given extra consideration. Post-column mixing becomes necessary when it is not possible to arrive at a mobile phase that satisfies both column and detector requirements (13). However, the technique involves the complications of an additional pump and mixing coil. Thus, when possible, direct electrochemical detection is preferred. Methanol and ethanol (common reverse phase solvents) are electrochemically oxidized at higher rates than water. Therefore, the background current (chromatographic baseline) for a given potential increases with the proportion of alcohol. With the methanol levels up to 55% used in this study, background current becomes noticeably higher about +0.8 V. At potentials above +1.0 V, the magnitude increases to around 10^{-8} amp. Above +1.2 V, water oxidation also becomes significant as current levels increase dramatically beyond this point for even purely aqueous solutions.

Initial experiments (e.g., Table 1) were carried out on carbon paste. The increased background currents at higher potentials and the physical effects of water-miscible solvents limit the use of carbon paste as a working electrode. Post-column addition of a second solution to dilute the alcoholic effluent and supply the necessary electrolyte for electrochemical detection with a carbon paste electrode has been reported (3). Recently, glassy carbon electrodes have become available. Glassy carbon works well as an alternative in such situations and was used for analyzing samples. This material is impervious to attack by solvents and requires only an occasional wiping with nonabrasive tissue (usually every few days) to clean the surface. Most compounds oxidize similarly on glassy carbon as compared to carbon paste. The response on a current density (current per unit area) basis is about the same for both electrodes, however, the noise is slightly higher on glassy carbon. As a result, the minimum detectable quantity is often higher on glassy carbon.

The solution resistance also can be a concern when large currents are encountered. The detector output current is generated by virtue of the interfacial potential of the working electrode causing electron transfer from (in this case) phenols in solution to the working electrode. When the current and/or solution resistance between the working and auxiliary electrodes is small, the potential controlled by the detector circuitry appears across the working electrode-solution interface. When either the resistance (R) or the current (I) is sufficiently large, the working electrode potential is significantly diminished by an amount equal to the product of R and I. High resistance can be caused by insufficient ionic strength. Large currents occur when large amounts of sample are injected onto the chromatograph resulting in a negative deviation in the response curve.

The large background current arising at high detector potentials influences normalized hydrodynamic voltammograms as illustrated in Fig. 1. In the region where background current increases significantly, the limiting current response begins to decrease. The low ionic strength of the mobile phase is responsible, in part, for this result. Increasing the buffer concentration relieves this effect; however, salts

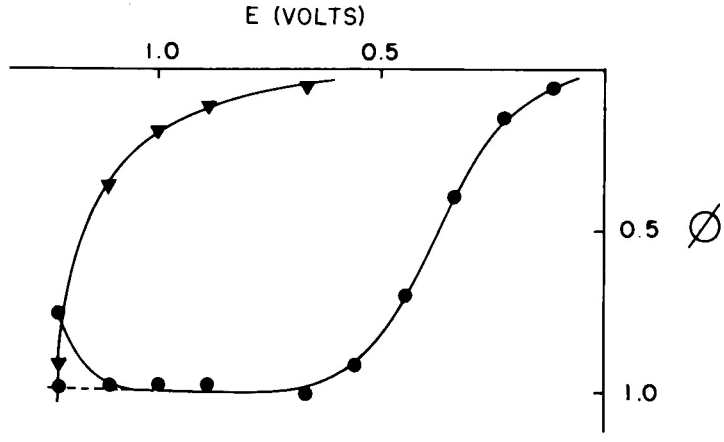

FIG. 1—Effect of I-R drop on the hydrodynamic voltammogram of BHA. Relative peak current response, ϕ, plotted vs. applied potential. (●——●), auxiliary electrode position downstream gives high cell resistance; (●- - -●), auxiliary opposite working in thin layer channel significantly reducing resistance; (▲——▲), background current, value at +1.2 V is 8.0×10^{-8} amp. Mobile phase: 0.01M phosphate buffer, pH 5.7, 55% methanol.

often contain traces of oxidizable impurities increasing the background current, negating the desired effect. In addition, the solubility of salts is limited with increased alcohol concentration. The physical placement of the electrodes is such that the current must pass along a portion of the thin layer detector channel. Appreciable resistance can be offered by this channel, because the solution film is 125 μm thick (or less, depending on the thickness of the Teflon spacer gasket). Two gasket sizes, 2 and 5 mil, are available. The channel resistance is decreased with the 5 mil gasket. Response, however, is roughly doubled for the 2 mil gasket compared with the 5 mil gasket. Modifying the cell geometry to place the auxiliary electrode opposite the working electrode reduces the current path to only the thickness of the spacer gasket. The resistance (R) for even low ionic strength mobile phases is then negligibly small. The influence of the background current on the response is eliminated (dashed portion in Fig. 1) and the linear portion of the calibration curve is dramatically extended. Detector cell geometry is discussed in detail elsewhere (14).

The background current associated with high potentials also contributes to baseline drift and higher noise. Figure 2 is a comparison of response for the same standard of TBHQ and BHA at +0.8 and +1.2 V. The most obvious difference is the baseline drift which arises from the slow adjustment of the electrode surface after the applied potential is changed. The

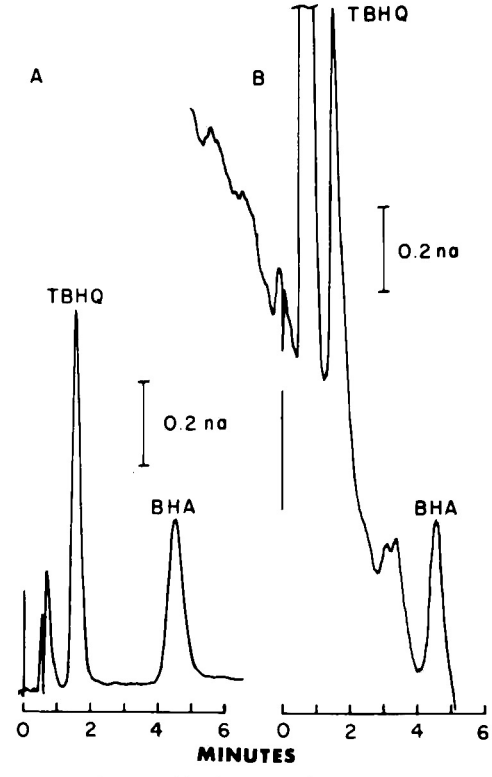

FIG. 2—Influence of high potential on baseline for standard solution of TBHQ and BHA, 2 ng of each injected. Glassy carbon electrode, TL-5A, 5 mil gasket. Mobile phase: 0.1M ammonium acetate, pH 6, 50% methanol. A, detector potential +0.8 V, background 1.5×10^{-9} amp; B, +1.2 V, 4.8×10^{-8} amp.

background current settles with time to a steady value. Thus, samples of very low concentration should be delayed until the detector has settled (usually 1–2 hr.).

Typical minimum detectable quantities for the antioxidants are well below 10^{-11} mole injected (e.g., Fig. 2). The low potentials required mean small background currents and carbon paste electrodes will operate satisfactorily. On the other hand, the glassy carbon working electrode with auxiliary electrode located in a close configuration is more widely applicable to the parabens and Ionox-100. The minimum detectability of the parabens is slightly greater due to the higher noise encountered. A typical value is 10^{-11} mole injected. The linear range using the proximate auxiliary electrode configuration can easily extend to 10^{-7} mole injected. In the case of parabens, however, this is not feasible due to electrode filming problems (see below). With the regular configuration, the break in linear response (although dependent on ionic strength, gasket thickness, etc.) occurs in the 10^{-9}–10^{-10} mole range.

Antioxidants

Extraction of oil- and fat-based samples spiked with BHA, TBHQ, and NDGA resulted in recoveries greater than 90% in all cases. BHA, TBHQ, and PG were determined by standard addition in several commercial food products. The levels ranged from 0.1 ppm in old samples to 500 ppm for cereal packagings. Recoveries of standard additions and spikes for BHA, TBHQ, and PG were greater than 85% in all cases with most in the range 92–102%. PG displayed little resolution ($k' < 1$) from the void volume response. Some of the food extracts yielded additional peaks but none presented any difficulty with quantitation of the analytes. The electrochemical detector provided lower limits of detectability compared with the UV detector. Roughly equal numbers of additional peaks (although not necessarily the same peaks) were encountered with both detectors.

Ionox-100 requires higher operating potentials to be detected and the following comments on the parabens are applicable for its determination. BHT was not detectable as evidenced by the absence of an oxidation wave in the voltammetry experiments.

Parabens

Operation at +1.1 V provided sufficient response and linear range for the parabens. They were determined in a group of 15 samples selected from a variety of cosmetic products (Table 2). The absolute recovery of the internal standard, EPHB, was 80% throughout.

Detector response over long term use (several hours) was diminished (typically <10%) by sample injection. The cause seemed to be adsorption of material (possible surfactants) onto the electrode surface. Injection of large amounts of parabens (>200 ng) can cause electrode filming by oxidation products which, in turn, will cause a decrease in subsequent response. As suggested by Ott (15), the frequent injection of a standard is recommended to follow changes in electrode efficiency. Filming is often a problem when oxidizing monophenols in traditional electrochemical experiments. The response to the parabens (and Ionox-100) was slightly less than that for the more easily oxidized antioxidants, however, it was significantly higher than for the UV detector (Fig. 3). This increased sensitivity may be used in 2 ways: The amount of sample can be reduced, or an extract incompatible with the chromatographic system may be diluted. For example, the benzoate procedure suffered from low extraction efficiences. Using ethanol has been shown to give higher recoveries (4). Although injection of ethanol is not entirely unacceptable, the resulting bands are distorted. A

Table 2. Types of samples analyzed[a]

Foods	Oils and fats	Cosmetics[b]
Instant cereals (BHA)	partially hydrogenated soybean oil, e.g., cooking and salad oils (BHA)	moisturizing lotions
Cereal packaging (BHA)	linseed oil[c]	protective creams (BPHB)
Snacks (BHA, TBHQ)	coconut oil[c]	makeup
Gelatin desserts (TBHQ)	baking shortening (BHA)	hydrophilic ointment lipstick (PG, TBHQ)
Baking pre-mixes (BHA)	partially hydrogenated animal fats, e.g., lard (PG)	shaving creams (MPHB only)
		antiperspirants (MPHB only)

[a] Compounds determined in parentheses.
[b] All contained MPHB and PPHB, additional analytes indicated in parentheses.
[c] No analytes originally present.

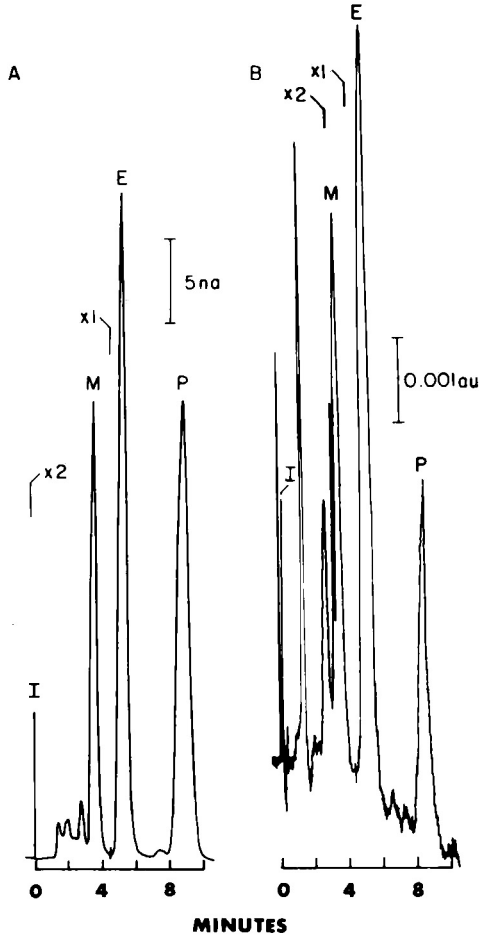

FIG. 3—Comparison of amperometric and ultraviolet detector response. Sample: extract of 1 year old cosmetic (Cover Girl makeup) containing MPHB and PPHB, with EPHB as internal standard, 55 ng injected. Glassy carbon working electrode TL-5 +1.1 V, 2 mil gasket, mobile phase same as in Fig. 2. Current scale indicated applies to ×1 portions of chromatograms. I = injection point, M = MPHB, E = EPHB, P = PPHB.

portion of the extract could be diluted in aqueous alcohol to alleviate the problem.

With no access to cosmetic samples intentionally formulated to exclude the benzoates, a suitable blank was unobtainable. Use of samples containing different benzoates is inappropriate. Different combinations of esters are used to accommodate the diversity in the chemical and physical properties of various samples. Fitzpatrick *et al.* (4) reported no interferences in UV absorbance detection of chromatographed formulations similar to the emulsions we analyzed. In addition, hydrophilic ointment (16) containing no benzoates gave no background response. Nevertheless, without adequate blanks or correlation with an accepted analytical method the values must only be considered as apparent analyte.

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TECHNICAL COMMUNICATIONS

Effect of Cupric Ions on the Analysis of Ethylenebis(dithiocarbamate) Residues in Tomato Juice

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Cupric ions interfered with the carbon disulfide evolution assay method for dithiocarbamate residues in tomato juice and in aqueous solution. Amounts of residue determined were reduced when $CuSO_4$ and a formulation of copper fungicide (Copper-Count-N) were present in the solutions analyzed.

Ethylenebis(dithiocarbamate) (EBDC) fungicides are used as protectants on tomato crops against such diseases as early blight (*Alternaria solani*), late blight (*Phytophthora infestans*), and anthracnose (*Collectotrichum phomoides*) (1), and may be found as residues in processed tomatoes (2–5). Their presence is currently determined by the CS_2 evolution method developed by Pease (6) and modified by Keppel (7) and Ripley and Simpson (8). Although it is nonspecific for individual dithiocarbamates, this method is rapid, sensitive, and relatively free of interferences. However, copper has been reported to interfere in the assay for dazomet residues (9), which is also based on the acid degradation of the dithiocarbamate and trapping of the CS_2 evolved by an ethanolic solution of cupric acetate and diethanolamine. The intensity of the yellow color of the cupric *N,N*-bis-(2-hydroxyethyl)dithiocarbamate which is formed is proportional to the amount of CS_2 evolved. The CS_2 is distilled from the digestion flask, so the only possible interference of the cupric ion would occur during the acid degradation. In the method for determining dazomet residues (9), only dilute HCl was used, whereas the current method for EBDC calls for the addition of $SnCl_2$ before hydrolysis. Because cupric ions are applied to tomatoes as fixed copper for the control of diseases such as bacterial speck (*Pseudomonas tomato*), bacterial canker (*Corynebacterium michiganense*), bacterial spot (*Xanthomonas vesicatoria*), and septoria leaf spot (*Septoria lycopersici*), we are concerned that their presence might interfere with the assay and be partially responsible for the apparent low recoveries of field-weathered residues (2–5, 10). Indeed, both types of fungicides are currently applied and sometimes combined to prevent diseases such as bacterial speck (11).

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Beckman DB. Read at 435 nm.

(b) *Carbon disulfide.* — Fisher spectranalyzed grade.

(c) *Ethylenediamine dihydrochloride.* — Matheson, Coleman and Bell, Norwood, OH 45212.

(d) $CuSO_4.5H_2O$.—Fisher certified ACS reagent grade.

(e) *Copper-Count-N.*—8% copper as cupric ammonium carbonate (Mineral Research and Development, Charlotte, NC).

(f) *Dithane-M45.*—Mancozeb, a complex of 80% manganese ethylenebis(dithiocarbamate), and zinc (Rohm and Haas Co. of Canada).

(g) *Disodium ethylenebis(dithiocarbamate).*—Prepare from CS_2 and ethylenediamine dihydrochloride (10).

Preparation of Sample

Tomatoes.—Juice of tomatoes which had been sprayed with various rates of Dithane-M45 were canned in 19 oz containers and stored frozen. Contents of several cans were thawed, pooled to ensure uniformity, and refrozen until use. Copper salts, 4 μg–50 mg A.I., were added directly to each 100 g sample before analysis.

Assay in water.—0.62 mg Dithane M-45 was added to 1 L distilled water with the required amount of copper. Fresh solutions were prepared for each assay and kept $\leq$6 hr.

Cupric ethylenebis(dithiocarbamate).—250 mL 1*M* $CuSO_4$ was added with vigorous stirring to solution of 50 g disodium ethylenebis(dithiocarbamate) in water. Resulting slurry was centrifuged at low speed, the supernate was discarded, and the pellet was resuspended in water. This was repeated 3 times, and then twice more with 95% ethanol. Residue was dried in a 50°C oven and pulverized in a ball mill.

Degradation of cupric EBDC.—1 mg cupric EBDC was added to 1 L water and 100 g subsamples were analyzed.

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Procedure

Samples were analyzed by the CS_2 evolution method as modified by Ripley and Simpson (8).

Results and Discussion

The recovery of EBDC expressed as mg mancozeb/kg in the presence of Cu^{++} is shown in Table 1. The recovery of EBDC was decreased when Cu^{++} was present in large amounts ($\geqslant$250 mg/kg). To ensure that these results would be applicable to fixed coppers used in agricultural practice, the experiment was repeated with Copper-Count-N as a source of copper. It was chosen as a typical commercial copper fungicide with a low amount of active ingredients. The results (Table 2), were more erratic in this case, and low recoveries were obtained with 20 mg Cu^{++}/kg, an amount that could be found at harvest if the copper fungicide was applied fairly close to harvest time.

In an attempt to identify the nature of this interference, the assay was repeated using water spiked with mancozeb as substrate. The results are shown in Table 3. Reproducibility was not as good because mancozeb is only sparingly soluble in water and 100 g subsamples contained some suspended material. This could be avoided by adding some ethylenediaminetetraacetic acid, but the introduction of the metal complexing agent could affect the recoveries. The results in Tables 1 and 3 tend to indicate that the influence cupric ions have on the degradation of EBDC is independent of the matrix.

The reduced recovery could possibly be due to the formation of a copper EBDC complex which would be more stable than mancozeb (12). The degradation of the preformed complex was then attempted. In 3 analyses, 0.68, 0.56, and 0.68 mg copper EBDC/kg were recovered, for an average recovery of 64%. While this is somewhat lower than for mancozeb, in the absence of copper (84%, Table 3), or with an equimolar amount of copper added (76–80%), it is higher than the recovery of EBDC in the presence of a large excess of copper.

Since the effect of Cu^{++} was much more dramatic with the commercial formulation, the emulsifiers it contains may interfere with the acid degradation of the EBDC. The composition of the formulation is not known, hence speculations would be irrelevant. This would not explain however the reduced recovery when $CuSO_4$ was used. It is important to note that then as much as 250 mg Cu^{++}/kg was required to reduce the recovery. Since the redox potential for the reaction $Sn^{+2} + 2Cu^{+2} \rightleftharpoons Sn^{+4} + 2Cu^{+1}$ is positive (13), the presence of Cu^{++} could counteract the beneficial effect of the reducing reagent, although in practice there should be a sufficient excess of $SnCl_2$ present (2 g). In an alternative procedure (14), the use of HI has been suggested to prevent the oxidation of EBDC by copper ions.

While the reasons for the interference of cupric ions with the assay for EBDC are not entirely clear at this point, it remains that the

Table 1. Effect of $CuSO_4$ on determination of EBDC in tomato juice

[Cu^{++}], mg/kg	EBDC, mg/kg[a]	Rec., %[b]
0	0.36±0.00	100
0.04	0.36±0.00	100
2	0.36±0.00	100
20	0.36±0.00	100
50	0.36±0.00	100
250	0.28±0.00	77
500	0.18±0.00	50

[a] As equivalent mancozeb based on triplicate sample with standard error.
[b] No Cu^{++} added is taken here as 100%.

Table 2. Effect of Copper-Count-N on determination of EBDC in tomato juice

[Cu^{++}], mg/kg	EBDC, mg/kg[a]	Rec., %[b]
0	0.39±0.04	100
0.04	0.39±0.02	100
0.4	0.40±0.00	103
2.0	0.41±0.00	105
20	0.30±0.08	77
50	0.17±0.02	44
250	–[c]	0
500	–[c]	0

[a] As equivalent mancozeb based on triplicate sample with standard error.
[b] No Cu^{++} added is taken here as 100%.
[c] None detectable.

Table 3. Effect of $CuSO_4$ on determination of EBDC in water spiked with 0.50 mg mancozeb/kg

[Cu^{++}], mg/kg	EBDC, mg/kg[a]	Rec., %[b]
0	0.42±0.04	84
0.04	0.38±0.06	76
0.4	0.40±0.04	80
2.0	0.38±0.05	76
20	0.38±0.05	76
50	0.39±0.05	78
250	0.13±0.01	26
500	0.09±0.00	18

[a] As equivalent mancozeb based on triplicate sample with standard error.
[b] Actual amount spiked is taken here as 100%.

presence of copper does reduce the recovery of CS_2. Copper residues are usually not a major concern on tomato crops, but it would seem appropriate to assay for them, if their presence is suspected, before assaying for dithiocarbamates. Further experiments to include field residues are planned. *In vitro* studies on the interaction between copper and ethylenebis(dithiocarbamate) fungicides have been carried out (15).

Acknowledgment

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High Pressure Liquid Chromatographic Determination of Naphthaleneacetamide Residues in Apples

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A simple, rapid, and accurate method has been developed for the determination of naphthaleneacetamide (NAAmide) residues in apples. After extraction with chloroform, separation is performed on an RP-8 high pressure liquid chromatographic column using acetonitrile-water (30 + 70) buffered to pH 3.5 at a flow rate of 1.7 mL/min. Either a variable wavelength ultraviolet detector set at 220 nm or a fluorometric detector can be used for quantitation. Average recoveries at the 0.01 and 0.1 ppm spiking levels were 83 and 89%, respectively. Incorporation into a previously described method for naphthaleneacetic acid (NAA) residues in apples has resulted in a procedure for the simultaneous determination of NAA and NAAmide.

Naphthaleneacetic acid (NAA), as its sodium salt, is used as a stop-drop agent on apples while naphthaleneacetamide (NAAmide) is used to induce fruit-thinning on apples early in the growing season (1, 2). A number of methods for determining NAA residues are available, and were summarized in a previous publication (2), but methodology for NAAmide residues is less common. Sigrist *et al.* (1) reported a fluorometric method for the simultaneous determination of NAA and NAAmide residues on apples with lower detection levels of 0.01 and 0.025 ppm, respectively. After extraction, the NAAmide residues were hydrolyzed at 90°C with

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2*N* NaOH and then determined as NAA. This paper describes attempts to expand our previously reported high pressure liquid chromatographic (HPLC) method for NAA (2) to include NAAmide. Two approaches were examined, the hydrolysis of the NAAmide to NAA (1) and the direct analysis of intact NAAmide residues.

METHOD

Apparatus

(a) *High pressure liquid chromatograph.*—Waters Associates Model ALC 202 equipped with Waters U6K universal injector (Waters Associates, Inc., Milford, MA 01757) and Schoeffel multiwavelength detector (Schoeffel Instrument Corp., Westwood, NJ 07675), set at 220 nm, and fluorescence detector operating at range 0.01 μamp, excitation 220 nm, and emission using band pass filter at 340 nm, connected in series.

(b) *HPLC columns.*—25 cm × 4.2 mm id packed with RP-8, 10 μm (Brownlee Laboratories) with 7.6 cm × 2 mm guard pre-column containing Co: Pell ODS, 25-37 μm (Whatman Inc., Clifton NJ 07014).

(c) *Ancillary equipment.*—As previously described (2).

Reagents

(a) *Mobile phase.*—0.8 g Na_2HPO_4 in 1 L acetonitrile-water (40+60) adjusted to pH 2.5 with phosphoric acid (hydrolysis studies), or 0.8 g Na_2HPO_4 in 1 L acetonitrile-water (30+70) adjusted to pH 3.5 (direct NAAmide analysis).

(b) *Standard solutions.*—0.5 mg NAAmide (Aldrich Chemical Co.)/mL acetonitrile and 0.2 mg NAA/mL mobile phase at pH 2.5.

(c) *Lithium hydroxide.*—Saturated solution in water (Fisher Scientific Co., Pittsburgh, PA 15219).

(d) *Solvents.*—As previously described (2).

Extraction

Extraction was carried out as described in ref. 2 except addition of 5 mL 10% H_2SO_4 was omitted. After base partitioning, using $NaHCO_3$, do not discard organic ($CHCl_3$) layer (contains NAAmide residues). Evaporate $CHCl_3$ layer (~ 225 mL) to 1–2 mL on rotatory evaporator, transfer to 50 mL round-bottom flask, and evaporate to dryness with stream of dry nitrogen. Dissolve residue in 2 mL (for 0.01 ppm determination) or 10 mL (0.1 ppm level) acetonitrile. Filter through Millipore filter type FH before injection.

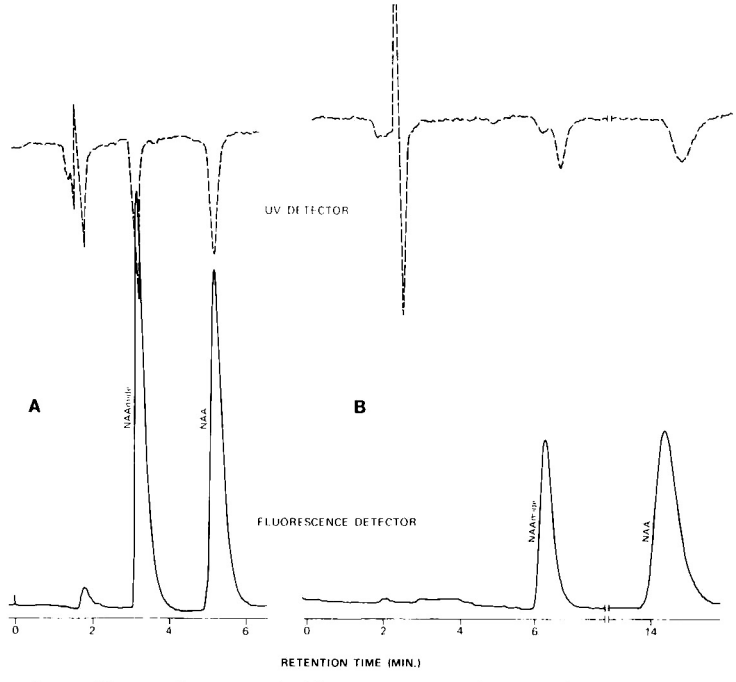

FIG. 1—Separation of 5 ng NAA and 5 ng NAAmide on an RP-8 column with solvent flow rate of 1.7 mL/min. A. Mobile phase acetonitrile-water (40 + 60) buffered to pH 2.5. B. Mobile phase acetonitrile-water (30 + 70) buffered to pH 3.5.

Determination

Inject 10 μL sample extract (either NAA or NAAmide extracts) onto HPLC column with mobile phase flowing at 1.7 mL/min. Compare peak areas and quantitate.

Results and Discussion

Before investigating the extraction and/or hydrolysis of NAAmide, a different HPLC column/solvent system was required; previously, a μBondapak C_{18} column and an acetonitrile-water (20+80) mobile phase buffered to pH 5.2 resulted in a 28 min retention time for NAAmide, with NAA appearing at 4 min. Using an RP-8 column and an acetonitrile-water (40+60) mobile phase buffered to pH 2.5, the retention times for NAAmide and NAA were 3.4 and 5.3 min, respectively (Fig. 1A). These HPLC conditions were used to study the extraction/hydrolysis approach to the quantitation of NAAmide. Initially, NaOH gave only partial conversion of NAAmide to NAA under reflux conditions using standards. Experiments with stronger bases such as KOH, potassium *tert*-butoxide, and LiOH showed a marked improvement in yields, with a saturated solution of LiOH under reflux for 1 hr giving quantitative conversion (i.e., 96–100%) to NAA. However, in trials with spiked apple samples recoveries varied from 55 to 95%, and, therefore, this approach was abandoned.

NAAmide was quantitatively extracted from apples using $CHCl_3$ but during the base partitioning of NAA into the aqueous layer, the NAAmide remained in the organic layer along with the majority of coextractives. This led to difficulties with the HPLC analysis due to the co-elution of NAAmide with interfering coextractives. A change in the mobile phase to acetonitrile-water (30+70) buffered to pH 3.5 resulted in a retention time of 6.2 min for NAAmide with NAA appearing at 14.2 min (Fig. 1B). As found previously (2), the fluorescence detector was 4 times more sensitive to NAA or NAAmide than was the UV absorbance detector. While no problems were encountered at the 0.1 ppm levels (Fig. 2A), interfering peaks observed with the UV detector hampered quantitation at the 0.01 ppm level (Fig. 2B). Recently, HPLC with fluorescence detection was

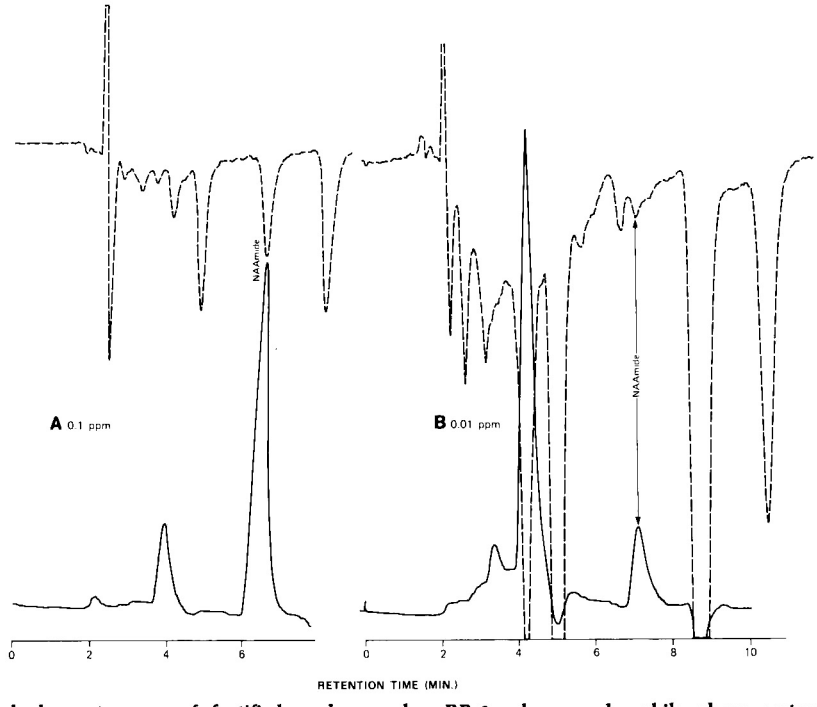

FIG. 2—Typical chromatograms of fortified apple samples. RP-8 column and mobile phase acetonitrile-water (30 + 70) at pH 3.5. A, 0.1 ppm NAAmide added; B, 0.01 ppm NAAmide added. (———) fluorescence detector; (– – –) UV detector.

Table 1. Recovery (%) of naphthaleneacetamide from apples

Sample	Added, ppm	
	0.1	0.01
1	89.5	81.8
2	96.0	81.1
3	85.3	85.8
4	86.1	86.6
5	89.1	79.6
Av.	89.2	83.0
Std dev.	4.8	3.1

used to determine NAA residues in citrus and processed products to a lower level of 0.008 ppm (3). In the present study, recoveries of NAAmide from spiked apple samples were in the range 83.0–89.2% (Table 1).

Also, it was found that both NAA and NAAmide could be determined simultaneously when present in the same apple sample. Due to the acid-base liquid partitioning step used for NAA and the fact that NAAmide remains in the $CHCl_3$ layer, final quantitation is performed on 2 separate fractions. Recoveries for NAA were as previously described (2), i.e., 90–95%, while NAAmide recoveries were similar to those shown in Table 1.

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Acknowledgments

The authors thank N. Looney, Agriculture Canada, Summerland, British Columbia, Canada for supplying apple samples.

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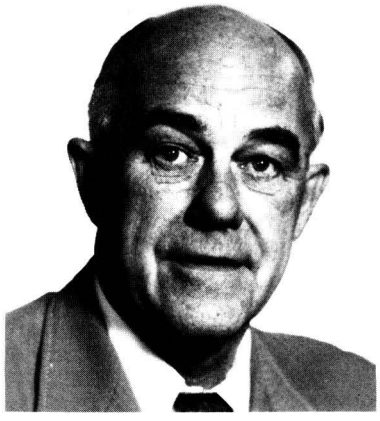
FOR YOUR INFORMATION



W. Perce McKinley, Named 1980 AOAC President

W. Perce McKinley, Director General of the Food Directorate, Health Protection Branch, Canada, is the 1980 AOAC President.

Dr. McKinley joined the Food and Drug Directorate in 1953 as a research scientist in endocrinology. In 1957 he was transferred to the Food Chemistry Section to work on pesticide residues. In 1964, he became Chief, Food Division, and, in 1965, Head of the Pesticide Section, Food Chemistry Division. In 1968, he was named Chief, Pharmacology and Toxicology Division, and in 1969, Director of Research, Food and Drug Research Laboratories. Finally, in 1972, Dr. McKinley was named the first Director General of the Food Directorate, Health Protection Branch, Canada.

Dr. McKinley was General Referee for Residues of Phosphated Insecticides and Miticides in Foods (1960–1966). He is the author of 68 publications on pesticide residue analysis; Dr. McKinley's Food Directorate staff are active participants in AOAC work.

Dr. McKinley holds the Bachelors of Science, Masters of Science and Ph.D. degrees from McGill University, Montreal. He is a member of the Canadian Institute of Food Science and Technology, Society of Toxicology, Institute of General Management, and the Canadian Association for Research in Toxicology. He resides in Stittsville, Ontario with his wife, Dorothy.

Mrs. Pierce Retires

Mrs. Rosalind Pierce, AOAC Business Manager for the past 33 years, will retire on January 18.

Nearly 80 friends and co-workers attended a retirement luncheon held in her honor on November 28 at the Ramada Inn in Rosslyn. Among those paying warm and sentimental tribute to her were former AOAC Executive Director William Horwitz, two former AOAC Presidents, Margarethe Oakley and Anthony J. Malanoski, former AOAC Executive Secretary Luther G. Ensminger, AOAC Treasurer Bernhard Larsen, AOAC consultant Morton Beroza, AOAC's new Assistant Executive Director George Schwartzman, and AOAC Executive Director David MacLean, who appointed Mrs. Pierce honorary General Referee in Culinary Arts and assigned her to oversee inter-kitchen collaborative studies for her recipes. She received a food processor as a going-away gift as well as a three-day vacation at the Homestead in Hot Springs, VA.

Mrs. Pierce, known to one and all as "Rosie," first came to the AOAC in 1946, and ran a one-woman office for then-Executive Director Dr. Heine Lepper in the South Agriculture Building in Washington, DC. She did everything—handled Journal subscriptions and book orders for *Official Methods of Analysis*, then in its sixth edition. She helped coordinate the Annual Meeting and wrote all appointment letters and correspondence for the General and Associate Referees.

Before coming to AOAC, she worked as a teacher, and, for a short while, with the Federal Bureau of Investigation Fingerprint Department. Educated at Marshall College (now University) in Huntington, West Virginia, and the University of Virginia in Charlottesville, she was teaching in the D.C. Public School system with Mrs. Heine Lepper, who suggested Rosie take the job with her husband, Dr. Lepper.

Born in Craigsville, Virginia, near Staunton, Rosie and her husband, Eddie, will now move to Staunton upon her retirement. She is wished the very best of luck by the entire staff of the AOAC.

Chemical Inventory Available From EPA

The Environmental Protection Agency has released the country's first comprehensive inventory of chemicals produced in the U.S. or imported here. The initial listing of chemical names includes 43,278 compounds, such as acids, alkalies, organic chemicals, plastics, and pigments produced or imported since January 1, 1975. A total of 7,420 chemical producers and importers reported for the inventory.

The inventory, required by the Toxic Substances Control Act, is not a compilation of suspect or dangerous chemicals but simply a listing of compounds manufactured or imported during the past four years. It will serve as a starting point for future EPA actions concerning human and environmental hazards. The Agency will update this listing periodically.

Firms wanting to produce or import chemicals not listed in the inventory must notify EPA in advance and submit available studies on the health and environmental effects of these new materials. EPA will provide one free copy of the inventory to each company or interested organization while supplies last. Copies are also available for inspection at EPA's ten regional offices and at public libraries around the country. Copies may be purchased for $34.50 from Superintendent of Documents, GPO, Washington, DC 20402, GPO No. 055-007-00004-7. A limited number of microfiche and computer tape copies are available from EPA. For further information, contact EPA Industry Assistance Office at 800-424-9065 or 202-554-1404 in Washington, DC.

USDA To Lease Biological Abstracts on Tape

The U.S. Department of Agriculture Science and Education Administration (SEA) has leased Biological Abstracts on Tape (BAT) from Bio-Sciences Information Service (BIOSIS), the world's largest English-language indexing and abstracting service for biological and biomedical research.

Biological Abstracts on Tape, the machine-readable version of the full English-language text of the abstracts appearing in Biological Abstracts (BA), is offered among a variety of library and information services available to USDA scientists, professional personnel, and formal cooperators. BAT was developed to complement BIOSIS Previews by providing textual support for the bibliographic and indexing information given for BA items in the BIOSIS Previews file.

Fluorescence Standard Available From National Bureau of Standards

The NBS Office of Standard Reference Materials announces the availability of Standard Reference Material (SRM) 936, Quinine Sulfate Dihydrate, for use as a fluorescence standard in the evaluation of methods and the calibration of fluorescence spectrometers.

A solution of 1.28×10^{-6} mol/L of SRM 936 in 0.105 mol/L perchloric acid is certified for its relative molecular emission spectrum, $E(\lambda)$, in radiometric units. The values of $E(\lambda)$ are certified at 5 nm intervals from 375 to 675 nm, for an excitation wavelength of 347.5 nm, and may be used to calculate the molecular emission spectrum in the various photon, radiometric, wavelength, and wavenumber units.

The certificate issued with SRM 936 provides instructions for making all necessary calculations. The full details of the preparation, certification, and use of this SRM are described in detail in NBS Special Publication 260–64.

SRM 936 is available from the NBS Office of Standard Reference Materials for $82 per 1 g unit. Orders for SRM 936 and requests for SP 260-64 should be addressed to the Office of Standard Reference Materials, Room B311, Chemistry Building, National Bureau of Standards, Washington, DC 20234.

1981 Meeting

July 20–24: International Conference on the Quality of Foods and Beverages—Recent Developments in Chemistry and Technology. The National Hellenic Research Foundation, Athens, Greece. Co-sponsored by the Agricultural and Food Chemistry Division of the American Chemical Society and the Society of Flavor Chemists, Inc., USA, in association with the Institute of Food Technologists, USA, the Department of Food Science and Technology of the University of Ioannina, Greece, and the Cereal Institute of Thessaloniki, Greece. For information, contact S. J. Kazeniac, Campbell Institute for Food Research, Camden, NJ 08101 (609/964-4000).

NEW PUBLICATIONS

NALCO Water Handbook. By Nalco Chemical Company, Frank N. Kemmer, Technical Editor. Published by McGraw-Hill, 1221 Avenue of the Americas, New York, NY 10020. 799 pages. Price $28.50.

This handbook is a compendium of information for both specialists and nonspecialists, providing comprehensive practical analyses of every aspect of water treatment in easy-to-understand, nontechnical language. Thorough coverage is given to adsorption, oil field flooding, current ion exchange practices, and scrubbers.

Biological Data in Water Pollution Assessment: Quantitative and Statistical Analyses (STP 652). American Society for Testing and Materials, 1916 Race St, Philadelphia, PA 19103. Soft cover. 193 pages. Price $17.50.

Topics in this publication range from statistical design of environmental studies to the use of multivariate analyses, ranking methods, diversity indices, and factor analyses.

1978 Indexes to EPA/NIH Mass Spectral Data Base and Volumes I–IV. By S. R. Heller, EPA, and G. W. A. Milne, NIH. Sponsored by the Environmental Protection Agency, Washington, DC 20460 and the National Institutes of Health, Bethesda, MD 20014. Price $65.00 per 5 part set, sold in sets only. SD No. 003-003-01987-9.

This publication presents a collection of 25,556 verified mass spectra of individual substances compiled from the EPA/NIH mass spectral file. The spectra are given in bar graph format over the full mass range. Each spectrum is accompanied by a Chemical Abstracts Index substance name, molecular formula, molecular weight, structural formula, and Chemical Abstracts Service Registry Number.

Trace Organic Analysis: A New Frontier in Analytical Chemistry. Edited by Harry S. Hertz and Stephen N. Chesler. Published by National Bureau of Standards, Washington, DC 20234. 788 pages. Price $14.00.

This volume is the proceedings of the 9th Materials Research Symposium held at the National Bureau of Standards in Gaithersburg, MD, April 10–13, 1978. Topics covered include Sampling and Sample Handling, State-of-the-Art Analytical Systems, Analytical Techniques on the Horizon, Analysis of Nutrients, Analysis of Organic Pollutants and Their Metabolites in the Ecosystem, Analysis of Drugs in Body Fluid, Analysis of Food Toxicants, and Analysis of Hormones and Neurotransmitters.

Safe Use of Pesticides. Third Report of the WHO Expert Committee on Vector Biology and Control. World Health Organization Technical Report Series, 1979, No. 634 (ISBN 92 4 120634 9). 44 pages. Price Sw. fr. 5. Order from WHO Publications Centre USA, 49 Sheridan Ave, Albany, NY 12210.

This booklet is the report of the WHO Expert Committee on Vector Biology and Control convened to advise on the safe use of pesticides in public health and agriculture and to review the latest toxicological data on new pesticides and new formulations. Annexes to the report describe the treatment of victims of pesticide poisoning and the techniques that may be used to collect samples from the places where poisoning occurs.

BOOK REVIEWS

Blood Drugs and Other Analytical Challenges. E. Reid (ed.). Methodological Surveys in Biochemistry, Vol. 7, Ellis Horwood, Chichester, UK, 1978. 355 pp. Price $47.50

This book is a collection of papers given at the Bioanalytical Forum at the University of Surrey in 1977. The major portion of the book concerns pharmaceutical analysis in biological tissue or fluid.

There are four major parts of the book. The first is an interesting and informative section which deals with an overview of methodology assessment and method development. The chapter by Curry and Whelpton, although far too limited, discusses the role of statistics in method development. They also discuss some of the well known problems of standard materials and internal standard measurement. The first part of the book concludes with a well deserved criticism of the present day chromatography terminology.

The second part of the book contains 5 chapters and is entitled "Subtle Gas Chromatography." It discusses the use of glass capillary GC and the use of electron capture detection for drug assay, as well as derivatization and flame ionization detectors. The chapter by Vessman is well documented with actual examples, and the chapter by Ehrsson discusses a limited series of derivatives for electron capture detection. A case history of bendiofluazide assay is included, however no statistical evaluation was done.

The third section of the book is devoted to mass spectrometric methods. The individual chapters discuss some actual examples; however, again no statistical evaluation of methods is usually provided. An interesting chapter by Frigerio discusses GC-MS as applied to dioxins at Seveso. The last chapter of the section, by Game *et al.*, is a forecast of possible HPLC-MS. The chapter is well written but no new work is presented; it is mainly a literature review through 1977.

The final section of the book is concerned mainly with HPLC, TLC, and other types of analysis.

A paper by Schill discusses ion-pair chromatography of drugs, and a great deal of actual data is included. Several other papers give actual assay protocol; however, in general no data for evaluation of a method are included. An extremely interesting paper by Bye discusses solid state sample preparation for LC.

Overall, the book is a useful reference for any analyst concerned with analysis of drugs in biological samples, many methods (mostly unofficial) are included, and the references are current through 1977. The major drawbacks of the book are a rather disconcerting manner of placing tables and illustrations in the middle of running text, and poor typesetting.

The book is highly recommended for anyone interested in analysis of drugs in tissue; however, the high price may limit the purchase to libraries or analytical groups.

WILLIAM P. VAN ANTWERP

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Evaluation and Optimization of Laboratory Methods and Analytical Procedures. D. L. Massart, A. Dijkstra, and L. Kaufman. Elsevier Scientific Publishing Company, PO Box 211, 100 AE Amsterdam, The Netherlands, 1978, 596 pp. $57.75.

This treatise contains much solid fundamental discussion of the basic attributes of analytical methods which should be mastered by all practitioners of analytical chemistry. It also contains many keen insights only vaguely perceived by scientists. Although this review will concentrate on the deficiencies from the reviewer's point of view, nevertheless, this volume is heartily recommended for reading and study by all who practice analytical chemistry.

First it must be pointed out that the subtitle is "A Survey of Statistical and Mathematical Techniques." As such, a good deal will have to be skipped by those not statistically or mathematically inclined or by those who find any equation containing a summation sign a formidable obstacle. Since the book is written for chemists, more interpretation of the equations and less "it is obvious that" or "clearly", and "therefores" is called for.

Second, as a mathematical and statistical survey, imprecise material is often treated so positively that the readers may be misled into forgetting that the attributes of methods are not constant physical characteristics attributable to methods. Accuracy, precision, sensitivity, specificity, and similar attributes are properties which have their own (variable) distributions arising from the abuse that methods and measurements are subjected to by analysts and laboratories.

Third, the influence of the analyst as a contributor to variability is largely ignored or confounded with laboratory variability. This is a polite professional fiction which must be overcome, if we are to improve our analytical capabilities. The analyst is usually the chief contributor to variability.

Fourth, the authors attempt to provide possible application of the mathematical techniques of

game theory, linear programming, supervised learning, etc. to analytical science. Such techniques are often applied to imprecise data. This variable input is handled by precise mathematical equations ("models" in the new linguistics). The final output is frequently assigned the precision of the mathematical manipulations, the imprecision of the input is overlooked.

Fifth, such techniques as cluster theory and classification techniques are applied to somewhat artificial situations such as the separation of 26 dyes or 22 sulfonamides. There may be situations where the theory is of assistance (e.g., handling the data from 226 liquid phases for chromatography), but usually the intuition and experience of the investigator places him within striking distance of the optimum without elaborate management techniques.

Sixth, it will probably come as a surprise to regulatory chemists that the authentic sample technique which they have been using to support legal actions for almost a century is a "supervised learning method." Their authentic samples are the training or learning set and the unknowns are the test set! The modern educational vocabulary has overflowed into analytical chemistry!

Finally, it should be pointed out that this is a difficult book to read. It is set by photographing a typewritten page, using a light impression on heavy paper. Cross references are frequent but there are no running page heads to guide the reader to his destination. It discourages bedtime use.

WILLIAM HORWITZ

Bureau of Foods
Food and Drug Administration
Washington, DC 20204

REMINDER: Deadlines

Nominations for the 1979 Fellows of the AOAC Award—March 3, 1980

Nominations for the 1979 Harvey W. Wiley Award—April 1, 1980

Nominations for the 1979–1980 Scholarship Award—May 1, 1980

For further information and nomination forms for Wiley Award, contact AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209

INSTRUCTIONS TO AUTHORS

Scope of Articles

The Journal of the AOAC will publish articles that present, within the fields of interest of the Association (*a*) unpublished original research; (*b*) new methods; (*c*) further studies of previously published methods; (*d*) background work leading to development of methods; (*e*) compilations of authentic data; (*f*) cautionary notes and comments on techniques, apparatus, and reagents; (*g*) invited reviews of methodology in special fields. All articles are reviewed for scientific content and appropriateness to the journal.

Preparation of Manuscript

Authors are required to submit three copies (one of which must be the original—ribbon—copy) of the complete manuscript, including all tables and all illustrations. The manuscript is to be typewritten on one side only of white bond paper, $8 \times 10\frac{1}{2}$ or $8\frac{1}{2} \times 11$ inches, with minimum page margins of 1 inch, and must be **double-spaced** throughout (including title, authors' names and addresses, footnotes, tables, references, and captions for illustrations, as well as the text itself). Tables are to be typed on separate sheets, *not* interspersed through the manuscript. Drawings and photographs should be mounted apart from the text or submitted as separate items, *not* interspersed through the text.

Style and Format

The text should be written in clear, concise, grammatical English. Unusual abbreviations should be employed as little as possible and must always be defined the first time they appear. Titles of articles should be specific and descriptive. Full first names, middle initial (if any), and last names of authors should be given. The address of the institution (including zip code) from which the paper is submitted should be given and should be in a form to which inquiries, proofs, and requests for reprints can be sent. Information supplementing the title and authors' names and addresses should be given in footnote form.

Methods, Results and/or Discussion, Acknowledgments, and Recommendations (applicable to reports of General and Associate Referees) should be placed in separate sections under appropriate headings typed in capitals and lower case letters, centered on the page, *not* underscored.

Abstracts: Each manuscript should be accompanied by a concise abstract (not more than 200 words). The abstract should provide specific information rather than generalized statements.

Introduction: Each article should include a statement on why the work was done, the previous work done, and the use of the compound being studied.

Methods: Methods should be written in imperative style, i.e., "Add 10 ml ... Heat to boiling ... Read in spectrophotometer." Special reagents and apparatus should be separated from the details of the procedure and placed in sections with appropriate headings; however, common reagents and apparatus (e.g., concentrated HCl, chloroform, ordinary glassware, ovens, etc.), or those which require no special preparation or assembly, need not be listed separately. Hazardous and/or carcinogenic chemicals should be noted. The steps of the procedure should not be numbered, but should be grouped together to form a logical sequence of two, three, or four operations. Any very long, detailed operation can be given in a separate section with an appropriate heading (e.g., Preparation of Sample; Extraction and Cleanup; Preparation of Standard Curve). Any necessary calculations should be included. Care should be taken that the number of significant figures truly reflects the accuracy of the method. Equations should be typed in one-line form. Wherever possible, metric units should be used for measurements or quantities.

Tables: All tables must be cited in the text consecutively. Tables are numbered by arabic numbers, and every table must have a descriptive title, sufficient so that the table can stand by itself without reference to the text. This title should be typed in lower case letters, *not* capitals, with the exception of the word "Table" and the first word of the descriptive portion of the title, of which the first letter is capitalized. Every vertical column in the table should have a heading; abbreviations may be used freely in the headings to save space, but should be self-evident or must be explained in footnotes. Footnotes to both the headings and the body of the table are indicated by lower case letters in alphabetical order; these letters should be underscored and raised above the line of type. Vertical and horizontal rules should be used sparingly; however, horizontal rules are used to bound the table at top and bottom and to divide the heads from the columns. Authors should refer to recent issues of the Journal for acceptable format of tables; tables should not exceed the normal page width of the Journal, and authors should attempt to revise or rearrange data to fit this pattern.

Illustrations: Illustrations, or figures, may be submitted as drawings or photographs. All figures must be cited in the text consecutively. Figures are numbered by arabic numbers, and all figures must be accompanied by descriptive captions, typed on one (or more) separate sheets, *not* on the figure itself. The figure should be identified by number on the back by a soft pencil or (preferably) a gummed label.

Drawings should be submitted either as the original drawing or a good glossy photograph; photocopies, multiliths, Verifax copies, Xerox copies, etc. are *not* acceptable. Drawings should be done in black

India ink (ordinary blue or blue-black ink is not acceptable) or with drafting tape on white tracing paper or tracing cloth or on "fade-out" graph paper (ordinary graph paper ruled with green or dark blue ink is not acceptable). Lettering should be done with a Leroy lettering set, press-on lettering, or a similar device; freehand or typewritten lettering is not acceptable. Values for ordinate and abscissa should be given, with proper identification conforming to journal style (example: wavelength, nm), at the sides and bottom of the figure. Lettering or numbering on the face of the figure itself should be kept at a minimum; supplementary information should be given in the caption. Several curves on the same figure should be identified by simple symbols, such as letters or numbers, and the proper identification or explanation given in the caption. Letters and numbers should be large enough to allow reduction to journal page or column size. *JAOAC* does not publish straight line calibration curves; this information can be stated in the text. The same data should not be presented in both tables and figures.

Footnotes: Footnotes are a distraction to the reader and should be kept to a minimum. Footnotes to the text are identified by arabic numbers set above the line of type (not asterisks or similar symbols). Each footnote must be indicated by its number within the text.

Acknowledgments: Essential credits may be included at the end of the text but should be kept to a minimum, omitting social and academic titles. Information on meeting presentation, financial assistance, and disclaimers should be unnumbered footnotes and appear after the *References* section.

References: References to previously published work should be collected at the end of the article under the heading "References." Each item in the list is preceded by an arabic number in parentheses. Every reference must be cited somewhere in the text in numerical order (rather than alphabetical or chronological). (*Note:* If an article contains only one reference, this reference may be inserted directly in the text, rather than placed at the end.) It is the author's responsibility to verify all information given in the references.

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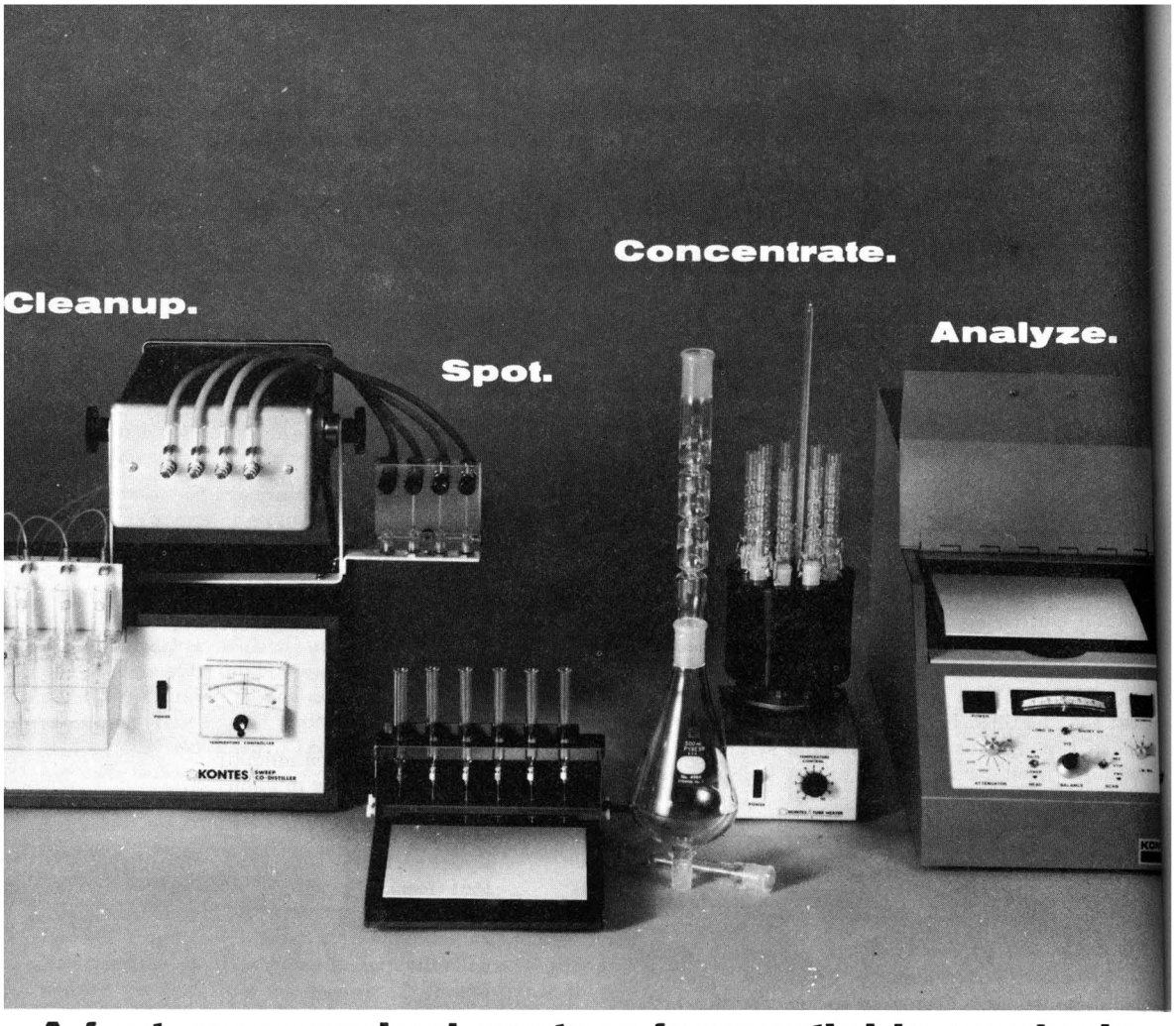
The compendium of methods of the Association should be listed as follows: *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, with appropriate section numbers; the edition and year are, of course, subject to change.

Symbols and Abbreviations

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

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



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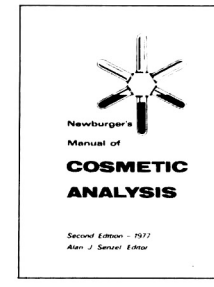
cosmetic analysis



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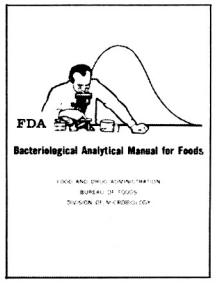

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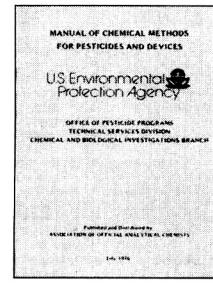

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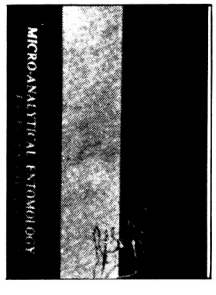

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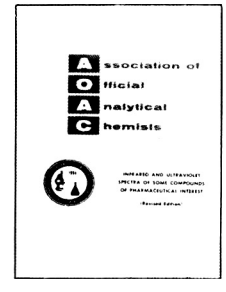

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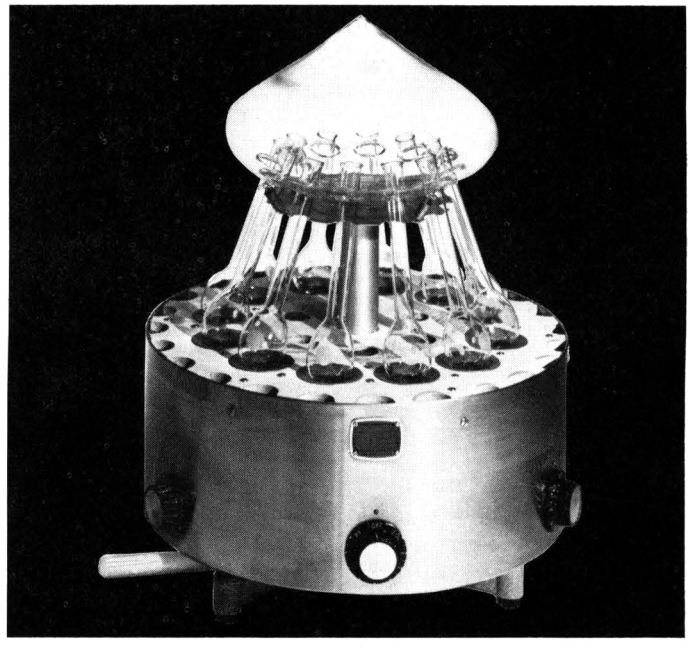

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