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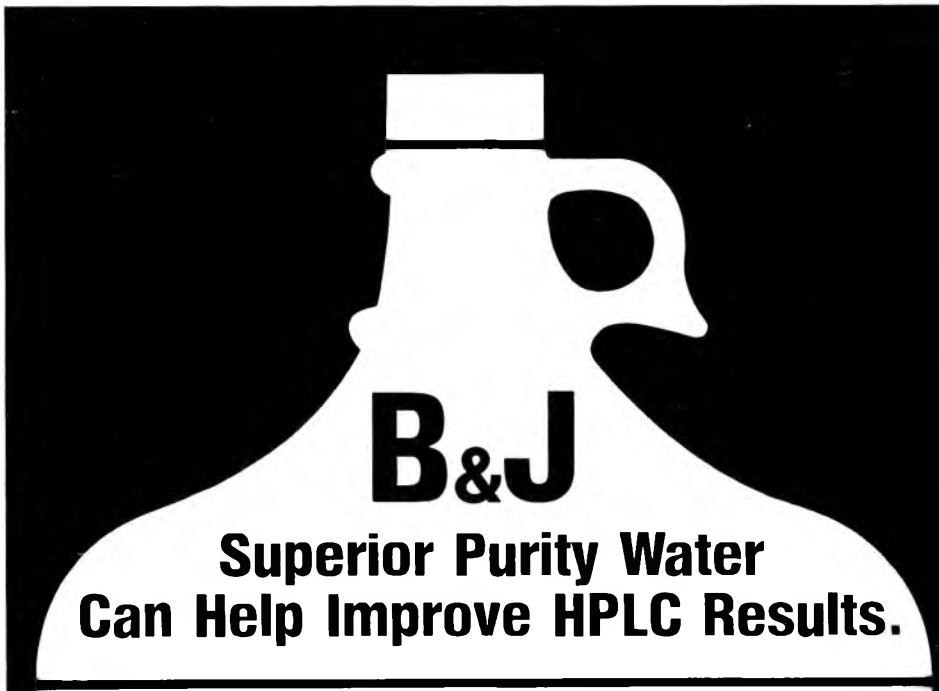

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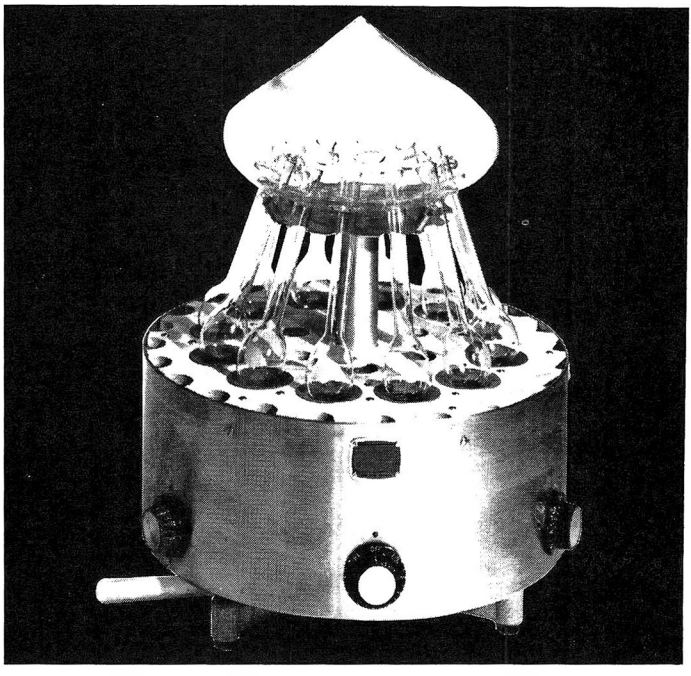

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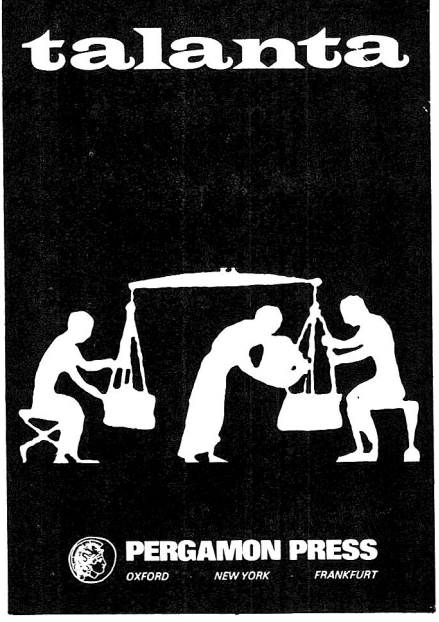

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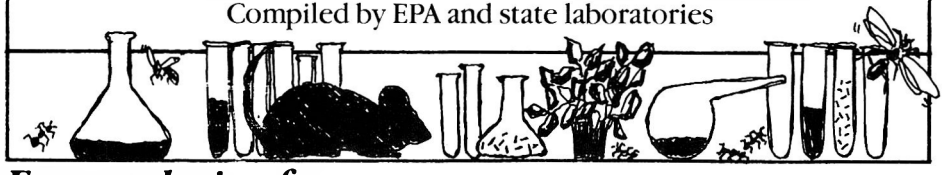

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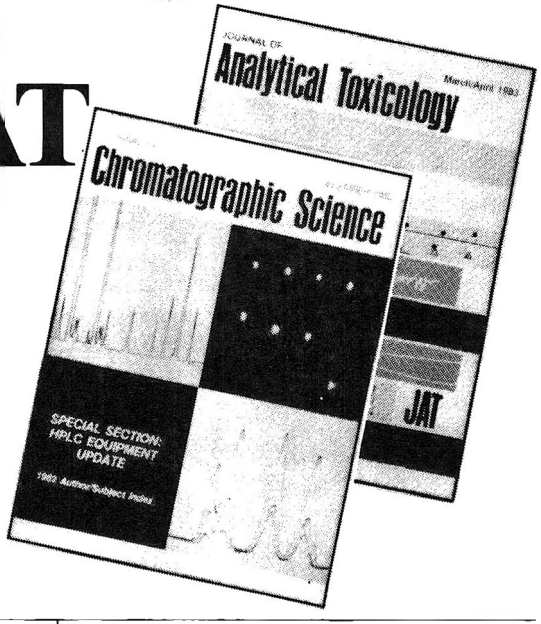

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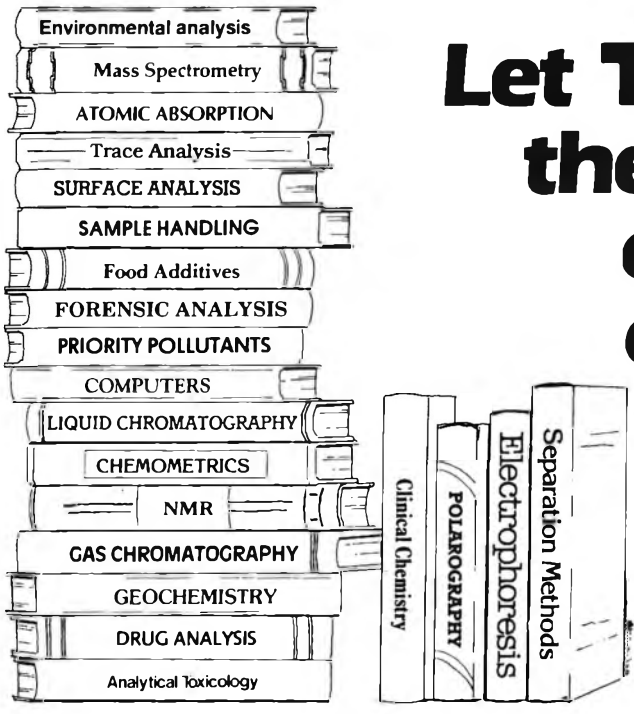

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Evaluation of a Testing Program for Aflatoxin in Corn

THOMAS B. WHITAKER and JAMES W. DICKENS
U.S. Department of Agriculture, Agricultural Research Service, North Carolina State University, Raleigh, NC 27650

A computer model that accounts for sampling and analytical variability was developed to simulate the aflatoxin testing program administered by the North Carolina Department of Agriculture (NCDA) to regulate aflatoxin in corn meal. Monte Carlo solution techniques were employed to account for conditional probabilities that rise from multiple samples being used in the testing program. The NCDA testing program was then evaluated by applying the computer model to a hypothetical group of 1000 corn meal lots with the same distribution of aflatoxin concentrations as was observed among aflatoxin assays made by NCDA on commercial lots of corn meal from 1977 to 1980. The average of the 1000 lots assayed was 17.7 parts per billion (ppb). The model predicted that 79.5% of the lots would be accepted and 20.5% of the lots would be rejected by the NCDA testing program. The accepted and rejected lots contained an average of 5.7 and 64.2 ppb aflatoxin, respectively. The testing program accepted 7.3% of the lots with more than 20 ppb aflatoxin (consumers' risk) and rejected 1.0% of the lots with 20 ppb or less (processors' risk). A correct decision was made 94% of the time.

The Food and Drug Protection Division of the North Carolina Department of Agriculture (NCDA) conducts a program to regulate aflatoxin in corn meal produced in North Carolina. Each lot of corn meal sampled in this program is tested according to the procedure diagrammed in Figure 1. A 4.54 kg sample from the lot is subdivided into five 50 g samples. The aflatoxin is solvent-extracted from the corn meal by the CB method (1). If the analysis of one sample is ≤20 parts per billion (ppb) total aflatoxin, the lot is accepted. Otherwise, 2 more samples are analyzed. If the difference between each of the 3 analyses and their average (A) is less than 0.3A, the lot is accepted when A is ≤25 ppb and the lot is rejected when A is >25 ppb. When any one of the 3 analyses is not within the range of (A ± 0.3A) ppb, the fourth and fifth samples are analyzed. The lot is accepted when the average of the 5 analyses is ≤25 ppb and the lot is rejected when the average is >25 ppb. Although the NCDA testing program has an action level of 25 ppb aflatoxin, the U. S. Food and Drug Administration has an administrative guideline of 20 ppb for aflatoxin in corn meal (2).

Because of the variability associated with testing corn for aflatoxin (3), analyses of samples from good lots may indicate that the lots are bad (processors' risk) and analyses of samples from bad lots may indicate that the lots are good (consumers' risk). The objective of this paper is to determine the processors' risk and the consumers' risk associated with the NCDA corn meal aflatoxin testing program.

Theory and Procedure

The total variance (error) associated with aflatoxin tests may include the variance in aflatoxin concentrations of replicated samples taken from the lot (sampling error), the variance in aflatoxin concentrations of replicated subsamples taken after comminution of the material in the sample (subsampling error) and the variance in replicated analyses of material with the same aflatoxin concentration (analytical error). The result of an aflatoxin test $\bar{x}$ may be represented by the following equation (4):

$$\bar{x} = \mu + \alpha + \beta + \gamma \qquad (1)$$

where μ is the true aflatoxin concentration in the lot tested, α is the random error due to sampling with expected value zero and variance C, β is the random error due to subsampling with expected value zero and variance F, and γ is the random error due to analysis with expected value zero and variance Q.

If there is both stochastic and functional in-

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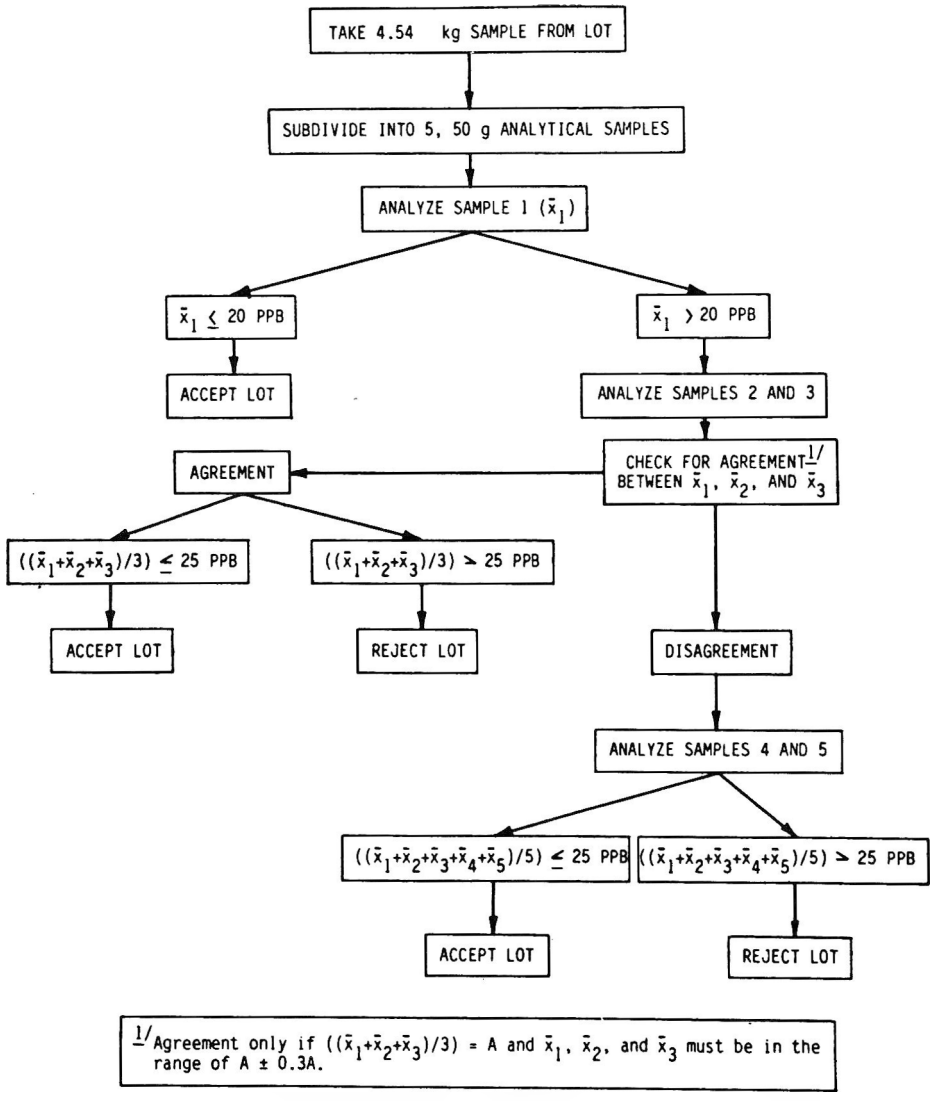

Figure 1. Diagram of North Carolina Department of Agriculture's aflatoxin testing program for corn meal.

dependence among the random errors in equation 1, the following variance relationship exists:

$$V = C + F + Q \qquad (2)$$

where V is the total variance of the test, C is the variance due to sampling, F is the variance due to subsampling, and Q is the variance in analysis. Because sample comminution is not used for the samples of corn meal before subdivision, the 50 g analytical samples used in the NCDA testing program are not different than 50 g samples taken directly from the lot. As a result, there is no subsampling variance ($F = 0$) and equation 2 reduces to the following variance relationship for the NCDA testing program:

$$v = c + q, \qquad (3)$$

where v is the total error of the test on lots of corn meal, c is the variance among 50 g samples of corn meal taken from the lot, and q is the variance among analyses of corn meal by the CB method. (Use of capital letters in equation 2 and small letters in equation 3 reflect population parameters and sample estimate of population parameters, respectively.) A previous study has developed the following equations for the variance C among 50 g samples of corn ground to

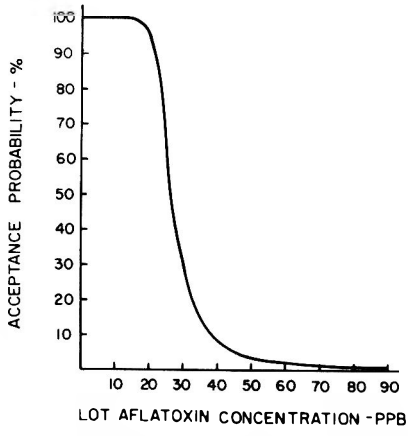

Figure 2. Operating characteristic curve of NCDA corn meal aflatoxin testing program.

pass a number 20 sieve and the variance Q among analyses by the CB method when the true aflatoxin concentration of the corn is μ (3):

$$C = 0.25\,\mu, \text{ and} \qquad (4)$$

$$Q = 0.07\,\mu^2. \qquad (5)$$

Use of Monte Carlo techniques for computerized simulation of aflatoxin testing programs has been described (5). Similar techniques and the variance estimates given by equations 4 and 5 were used to determine the probability of accepting corn meal lots with specified aflatoxin concentrations when the NCDA testing program diagrammed in Figure 1 is used. Simulated aflatoxin tests were made on 2000 lots with a given aflatoxin concentration μ. The probability of accepting lots ($P\mu$) with that aflatoxin concentration (μ) was then computed by dividing the number of lots accepted by 2000. The above procedure was repeated over a range of aflatoxin concentrations.

The number of lots with a given aflatoxin concentration that will be accepted ($L\mu$) by the NCDA aflatoxin testing program may be computed with the following equation:

$$L\mu = TL \times D\mu \times P\mu \qquad (6)$$

where TL is the total number of lots of corn meal tested, $D\mu$ is the fraction of the lots at a given aflatoxin concentration μ, and $P\mu$ is the probability of accepting a lot with an aflatoxin concentration μ. The distribution of corn meal lots according to aflatoxin concentrations ($D\mu$) was approximated by using the distribution of NCDA test results on 947 lots of corn meal tested from 1977 to 1980.

Table 1. Number of lots with indicated aflatoxin concentrations accepted and rejected by current NCDA corn meal aflatoxin testing program

Lot concn,	No. of lots		
ppb	Tested	Accepted	Rejected
0	350	350	0
1	60	60	0
2	40	40	0
3	30	30	0
4	20	20	0
5	20	20	0
6	20	20	0
7	20	20	0
8	18	18	0
9	17	17	0
10	16	16	0
11	16	16	0
12	15	15	0
13–14	27	27	0
15–16	26	26	0
17–18	23	23	0
19–20	21	20	1
21–22	20	17	3
23–24	19	13	6
25–26	16	9	7
27–29	21	8	13
30–34	32	7	25
35–39	25	3	22
40–44	17	0	17
45–49	14	0	14
50–59	20	0	20
60–69	15	0	15
70–79	10	0	10
80–89	10	0	10
90–99	10	0	10
≥100	32	0	32
Totals	1000	795	205

Results and Discussion

The computed acceptance probability ($P\mu$) for the NCDA testing program is plotted in Figure 2. The curve, frequently called an operating characteristic (OC) curve, shows that the NCDA testing program will accept nearly all lots ($P\mu \cong 100\%$) with less than 19 ppb aflatoxin and will reject nearly all lots ($P\mu \cong 0\%$) with more than 90 ppb aflatoxin.

Calculated numbers of lots tested, accepted, and rejected at various lot aflatoxin concentrations are given in Table 1. The total number of lots to be tested (TL) was estimated as 1000, the number of lots tested at each concentration was calculated as $D\mu \times 1000$, the number of lots accepted at each concentration was calculated as $D\mu \times 1000 \times P\mu$, and the number of lots rejected at each concentration was calculated as $D\mu \times 1000 \times (1 - P\mu)$.

Table 2 summarizes the results from Table 1. Of the 1000 lots tested, 73.9% were good ($\mu \leq 20$ ppb) and 26.1% were bad ($\mu > 20$ ppb). The av-

Table 2. Number of lots accepted and rejected and aflatoxin concentration in different portions of lot population

Item	Good ($\mu \leq 20$ ppb)	Bad ($\mu > 20$ ppb)	Total
No. of lots accepted	737*	58	795
Av. aflat. concn (ppb)	4.1	26.4	5.7
No. of lots rejected	2	203*	205
Av. aflat. concn. (ppb)	19.2	64.5	64.2
Total no. of lots	739	261	1000
Av. aflat. concn (ppb)	4.1	56.1	17.7

* Correct decision was made for these lots.
Correct decision = (737 + 203) (100)/1000 = 94%
Consumers' risk = % accepted lots with >20 ppb aflatoxin: 58(100)/795 = 7.30%
Processors' risk = % rejected lots with ≤20 ppb aflatoxin: 2(100)/205 = 0.98%
Average number of assays = 1.74

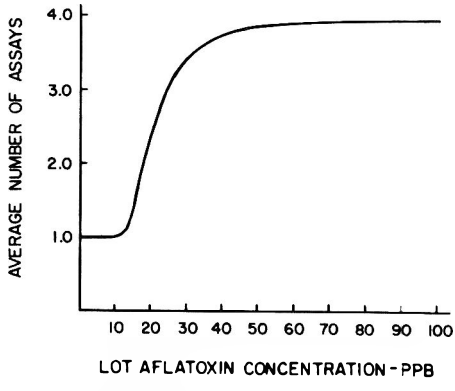

Figure 3. Average number of subsamples assayed for a decision at various lot concentrations.

erage aflatoxin concentrations in the good and bad lots tested were 4.1 and 56.1 ppb, respectively. Of the 739 good lots tested, 737 lots were correctly accepted and 2 were incorrectly rejected (processors' risk) by the testing program. Of the 261 bad lots tested, 203 lots were correctly rejected and 58 lots were incorrectly accepted (consumers' risk). Overall, the correct decision was made 94.0% of the time. The consumers' and processors' risks were 7.3 and 1.0%, respectively.

A plot of the average number of 50 g samples assayed per lot at various aflatoxin concentrations is shown in Figure 3. At low lot concentrations, only one assay per lot was used to make a decision. As the lot concentration increased, the number of sample assays per lot increased and leveled off at about 3.95. The average number of sample assays per lot for the 1000 lots was 1.74.

The results of this evaluation procedure reflect the particular lot distribution chosen. Lot distributions according to aflatoxin concentrations and the corresponding risks to the processor and consumer will vary from year to year and from one production location to another within a given crop year.

Acknowledgment

The authors thank the Food and Drug Protection Division of the North Carolina Department of Agriculture for information and assistance provided in this study.

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Dilution Errors in Aflatoxin Determinations Caused by Compounds Extracted from Peanuts

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Several methods have been developed to analyze peanuts for aflatoxin by using thin layer chromatography (TLC). These methods depend on solvent extraction of aflatoxin from a sample of the product. Unfortunately, solvent solutions used to extract aflatoxin from peanuts also extract measurable quantities of other compounds such as oils, fats, sugars, and protein. The volume of these extracted compounds causes error in measuring the proportion of the solvent solution analyzed for aflatoxin. Also, because the cleanup procedures for some methods are inadequate, the volume of some of these extracted compounds also causes error in measuring the proportion of the extracted aflatoxin placed on TLC plates. These 2 errors cause underestimation of aflatoxin concentrations by approximately 11, 14, and 5% for the CB method, the modified version of the BF method generally used for raw peanuts, and a water slurry method, respectively. The correction specified by the CB method for fats in the extraction solvent reduces the approximate error for the CB method from 11 to 1%.

Several methods have been developed to analyze peanuts for aflatoxin by using thin layer chromatography (TLC). Brief summaries for the CB method (1), the AMS method (2), and the water slurry (WS) method (3) are given in Table 1. Because filtration of chloroform extract from raw peanuts is extremely difficult, the extract was centrifuged instead of filtered as specified by the CB method. Substitution of centrifugation for filtration was proposed by Waltking (4). The AMS method is a modification of the BF method (1). The WS method has been modified from the published WS method by adding a filtration step in the cleanup procedure.

The above methods require solvent extraction of aflatoxin from a sample of the product. A measured proportion of the extraction solvent is subjected to cleanup procedures to remove some of the other compounds extracted along with the aflatoxins, and solvents used in the cleanup procedures are evaporated leaving a residue containing the aflatoxin. The residue is dissolved in a dilution solvent and the amount of aflatoxin in a measured proportion of dilution solvent is determined by placing it on a TLC plate. The aflatoxin concentration of the sample in nanograms of aflatoxin per gram of peanuts or parts per billion (ppb) may then be calculated by the following equations:

$$ng/g = A/G, \quad \text{and} \qquad (1)$$

$$G = EDW \qquad (2)$$

where A = ng aflatoxin in the amount of dilution solvent placed on the TLC plate, G = g peanuts represented by the dilution solvent placed on the TLC plate, E = proportion of extraction solvent subjected to the cleanup procedure, D = proportion of dilution solvent placed on the TLC plate, and W = weight in g of the sample.

Unfortunately, the volume of extracted compounds other than aflatoxin causes error in measurement of the proportion of extraction solvent subjected to the cleanup procedure (E). Because the cleanup procedure is inadequate, the residue from these extracted compounds also causes error in measurement of the proportion of dilution solvent placed on the TLC plate (D). The objective of this study was to determine the magnitude of these 2 errors for the CB method, the AMS method, and the WS method when they are used for raw peanuts.

Experimental

Approximately 7 kg raw peanuts was comminuted in a subsampling mill (5) and the comminuted material was thoroughly blended. Samples of this material were used for the study.

Extraction Solvent Error

CB method: Twenty 50 g samples of comminuted peanuts were carried through Step 2 of the CB method outlined in Table 1. A 50 mL portion of the chloroform extract for each sample was transferred from the centrifuge bottle to a 250 mL filtering flask. The portion was dried under vacuum on a steam table. A 50 mL portion of chloroform measured with a 50 mL volumetric

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Table 1. Brief summary of CB, AMS, and WS methods for aflatoxin analysis in peanuts

CB Method:
1. Extract 50 g sample of comminuted peanuts with 250 mL chloroform.
2. Separate chloroform extract solution by centrifuging. (Published method specifies filtration instead of centrifugation.)
3. Use 50 mL chloroform extract solution on sodium sulfate–silica gel column for cleanup.
4. Elute aflatoxin with chloroform–methanol.
5. Evaporate eluate, leaving residue which contains aflatoxin from 50 mL chloroform extract solution.
6. Dissolve residue with dilution solvent and place measured proportion of solvent on TLC plate for aflatoxin quantitation.

AMS Method:
1. Extract 1100 g comminuted peanuts with 3 L methanol–water (55 + 45) and 1 L hexane.
2. Separate methanol–water extract solution by centrifuging.
3. Wash 50 mL methanol–water extract solution with 50 mL chloroform.
4. Evaporate chloroform, leaving residue which contains aflatoxin from 50 mL methanol–water extract solution.
5. Dissolve residue with dilution solvent and place measured proportion of solvent on TLC plate for aflatoxin quantitation.

WS Method:
1. Blend 1100 g comminuted peanuts with 1.5 L water and 22 g sodium chloride.
2. Blend 130 g slurry from Step 1 with 180 mL methanol and 60 mL hexane.
3. Separate methanol–water extract solution by centrifuging.
4. Filter methanol–water extract solution (71% methanol by volume) through coarse filter paper and dilute 50 mL filtrate with 25 mL 10% sodium chloride solution.
5. Wash 75 mL diluted solution with 50 mL chloroform.
6. Evaporate chloroform, leaving residue which contains aflatoxin from 50 mL portion of methanol–water extract solution.
7. Dissolve residue with dilution solvent and place measured proportion of solvent on TLC plate for aflatoxin quantitation.

flask was used to wash the filtering flask. The solution was then returned to the 50 mL volumetric flask. A graduated pipet was used to measure the volume of solution in excess of 50 mL. The average loss from the 50 mL portion of chloroform during transfer between the flasks was estimated by making 5 measurements with clean flasks. The sum of the estimated average loss of 0.90 mL and the volume of excess solution measured with the graduated pipet was recorded as the volume of extracted compound.

AMS method: An 1100 g sample of comminuted peanuts was carried through Step 1 of the AMS method outlined in Table 1. Portions of the blended material were placed in sixteen 250 mL centrifuge bottles and centrifuged. A 50 mL portion of the methanol-water extract was transferred from each centrifuge bottle to a 250 mL filtering flask. Each portion was dried under vacuum on a steam table. A 50 mL portion of methanol–water (55% methanol by volume) measured in a 50 mL volumetric flask was used to wash the filtering flask. The solution was then returned to the 50 mL volumetric flask, and the volume of excess solution was measured with a graduated pipet. The average loss from the 50 mL portion of 55% methanol solution during transfer between the flasks was estimated by making 5 measurements with clean flasks. The sum of the estimated average loss of 0.43 mL and the volume of excess solution measured with the graduated pipet was recorded as the volume of extracted compound.

WS method: An 1100 g sample of comminuted peanuts was carried through Step 1 of the WS method outlined in Table 1. Twelve 130 g portions of the slurry was then carried through Steps 2 and 3. A 50 mL portion of the methanol–water extract was transferred from each centrifuge bottle to a 250 mL filtering flask. The portion was dried under vacuum over a steam table. A 50 mL portion of methanol–water (71% methanol by volume) measured in a 50 ml volumetric flask was used to wash the filtering flask. The solution was then returned to the 50 mL volumetric flask, and the volume of excess solution was measured with a graduated pipet. The average loss from the 50 mL portion of 71% methanol solution during transfer between the flasks was estimated by making 5 measurements with clean flasks. The sum of the estimated average loss of 0.43 mL and the volume of excess solution measured with the graduated pipet was recorded as the volume of extracted compound.

Dilution Solvent Error

CB method: Twenty 50 g samples of comminuted peanuts were carried through Step 5 of the CB method. The total weight of each vial containing the residue was determined. The vials were then washed with chloroform to remove the residue and reweighed. Weights were measured to the nearest 0.1 mg, and the weights of the residue were determined as the difference in the 2 weights.

AMS method: An 1100 g sample of comminuted peanuts was carried through Step 1 of the AMS method. Equal portions of the blended material were placed in eighteen 250 mL centrifuge bottles and centrifuged. Each of the 18 portions was then carried through Step 4 of the AMS method. The weight of residue in each of the 18 vials was determined according to the method outlined for the CB method.

Table 2. Summary of averaged data and calculations related to dilution errors in aflatoxin determinations caused by compounds extracted from peanuts by 3 analytical methods

Line	Description	Unit	Analytical method		
			CB	AMS	WS
1	Total volume of extraction solvent used	mL	250.0	3000.0	254.4
2	Volume of extraction solution subjected to cleanup procedure (analytical portion)	mL	50	50	50
3	Volume of extracted compounds in analytical portion	mL	4.560	0.863	0.499
4	Actual volume of extraction solvent in analytical portion (Line 2 − Line 3)	mL	45.440	49.137	49.501
5	Incorrect value of *E* normally used in Equation 2 to calculate *G* (Line 2/Line 1)	—	0.2000	0.0167	0.1965
6	Correct value of *E* (Line 4/Line 1)	—	0.1818	0.0164	0.1946
7	Percentage error in *E* caused by volume of extracted compounds [100 (Line 5 − Line 6) ÷ Line 6]	%	+10.011[a]	+1.829	0.976
8	Weight of residue from analytical portion after cleanup procedure	mg	5.50	41.54	13.43
9	Volume of residue (Line 8 ÷ 0.93781 mg/μL)	μL	5.86	44.30	14.32
10	Volume of dilution solvent used to dissolve residue	μL	300	300	300
11	Volume of solution placed on TLC plate	μL			
12	Actual total volume of diluted solution (Line 10 + Line 9)	μL	305.86	344.30	314.32
13	Incorrect value of *D* normally used in Equation 2 to calculate *G* (Line 11/Line 10)	—	0.0200	0.0200	0.0200
14	Correct value of *D* (Line 11/Line 12)	—	0.0196	0.0174	0.0191
15	Percentage error in D caused by the volume of residue dissolved in the dilution solvent [100 (Line 13 − Line 14) ÷ Line 13]	%	+2.041	+14.943	+4.712
16	Weight of peanuts in sample	g	50.0	1100.0	54.5
17	Incorrect value of *G* calculated with Equation 2 (Line 5) × (Line 13) × (Line 16)	g	0.200	0.367	0.214
18	Correct value of *G* calculated with Equation 2 (Line 6) × (Line 14) × (Line 16)	g	0.178	0.314	0.203
19	Incorrect concentration of aflatoxin calculated with Equation 1 (*A*/Line 17)	ppb	5.000*A*	2.725*A*	4.673*A*
20	Correct concentration of aflatoxin calculated with Equation 1 (*A*/Line 18)	ppb	5.618*A*	3.185*A*	4.926*A*
21	Percentage error in calculated concentrations of aflatoxin caused by using incorrect values of *G* in Equation 1 [100 (Line 19 − Line 20) ÷ Line 20]	%	−11.0[a]	−14.4	−5.1

[a] An adjustment in calculations specified by CB method will correct error caused by extracted compounds in analytical portion.

WS method: An 1100 g sample of comminuted peanuts was carried through Step 1 of the WS method. Nineteen 130 g portions of the slurry were then carried through Step 6 of the WS method. The weight of residue in each of the 19 vials was determined according to the method outlined for the CB method.

Measurement of Density of Extracted Compounds (Residue)

A large number of vials containing residue from routine analyses of peanuts by using the AMS method were accumulated. These vials were washed with chloroform and the chloroform solution was placed in a 500 mL beaker. The chloroform was evaporated on a steam table and the weight of 10 mL of residue at ca 20°C was determined by weighing a 10 mL volumetric flask before and after placing the residue in the flask. The weighings were made to the nearest 0.1 mg.

Results and Discussion

The residue after evaporating the chloroform in Step 4 of the AMS procedure resembled peanut oil. Ten mL residue weighed 9.3781 g, so the density of the material was 0.93781 g/mL at 20°C. This density is slightly more than the 0.914 g/mL density reported for peanut oil at 15°C (6). A mixture of 10 mL residue and 90 mL benzene–CH_3CN (98 + 2) totaled 100 mL, so the volumes of residue and dilution solvent are additive.

The average volume of extracted compounds in the 50 ml analytical portions of the extraction solvents were 4.560, 0.863, and 0.499 mL, respectively, for the CB, AMS, and WS methods

(Table 2, line 3). Because of the volume of these extracted compounds, the value of E generally used to compute aflatoxin concentrations is in error by +10.0, +1.8, and +1.0%, respectively, for the CB, AMS, and WS methods (Table 2, line 7). However, an adjustment in calculations to account for 5 mL of these extracted compounds has been specified for the CB method (1).

The average volumes of residue in the dilution solvents were 5.86, 44.30, and 14.32 μL, respectively, for the CB, AMS, and WS methods (Table 2, line 9). The errors in D caused by this residue when 300 μL dilution solvent was used were +2.0, +14.9, and +4.7%, respectively, for the CB, AMS, and WS methods (Table 2, line 15). Without correction for errors in D and E, the errors in computed aflatoxin concentrations were −11.0, −14.4, and −5.1%, respectively, for the CB, AMS, and WS methods (Table 2, line 21). The correction specified by the CB method for fats in the extraction solvent will reduce the error for this method from −11.0% to approximately −1.0%. Corrections for the volume of extracted compounds and dilution errors probably should be specified for each product analyzed by these methods.

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VITAMINS AND OTHER NUTRIENTS

Reverse Phase High Pressure Liquid Chromatographic Determination of Vitamin K_1 in Infant Formulas

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A reverse phase high pressure liquid chromatographic (HPLC) method for quantitating vitamin K_1 in enzymatic hydrolysates of infant formula is described. The vitamin is extracted with *n*-pentane before determination by isocratic and isothermal reverse phase HPLC. Recovery of vitamin K_1 added to 5 infant formulas ranged from 84 to 103%.

The Infant Formula Act of 1980 requires that infant formulas be analyzed for vitamin K_1 (1). There is no standardized or generally accepted method of determining vitamin K_1 in infant formulas. The bioassay method (2, 3) employed for the determination of vitamin K_1 in blood plasma is time-consuming and requires the maintenance of a battery of chicks. It is therefore not practical as a routine method. Manes et al. (4) described a chemical method for vitamin K_1 determination, in which the vitamin is separated from lipids using open column absorption chromatography followed by detection and quantitation using thin layer reflectance densitometry. Although this method was developed for the analysis of infant formula, it did not yield reproducible results in this laboratory. Barnett et al. (5) used enzymatic hydrolysis to degrade lipids in infant formula samples, followed by separation of vitamin K_1 from other oil-soluble vitamins by reverse phase liquid chromatography. The method of Barnett et al. did not produce meaningful data in this laboratory because of problems encountered during liquid chromatographic separation.

This paper describes modifications to the Barnett et al. method that allow routine and accurate chromatographic separation of vitamin K_1 in infant formulas, while retaining the enzymatic hydrolysis of lipids to reduce interferences.

Experimental

Reagents and Apparatus

(a) *Buffer solution.*—pH 7.7, 0.2M monobasic sodium phosphate–0.2M dibasic sodium phosphate (10.5 + 90.5) diluted to 200 mL with water.

(b) *Mobile phase.*—Methanol–acetonitrile–tetrahydrofuran–water (39 + 39 + 16 + 6). Methanol and acetonitrile were high pressure liquid chromatographic (HPLC) grade (Fisher Scientific Co.). Tetrahydrofuran was ultraviolet (UV) grade (Burdick and Jackson). Water was deionized and distilled.

(c) *Hexane.*—UV grade (Burdick and Jackson).

(d) *Liquid chromatograph.*—Waters Associates Model 244 with M-6000 solvent delivery system, U6K injector, and Model 440 detector equipped with filters to operate at 254 nm. Column: 25 cm × 4 mm stainless steel, slurry-packed with μBondapak C_{18}. Flow rate 1.5 mL/min (ca 1000 psi); elution at ambient temperature under isocratic conditions. Omni-Scribe recorder (Houston Instrument) is adjusted to give full scale reading at 0.02 absorbance unit. Chart speed 0.5 cm/min.

Standard Preparation

Stock solution.—Accurately weigh 25 mg vitamin K_1 (Grand Island Biological Co.) and transfer to 100 mL volumetric flask. Dilute to volume with hexane. *Working standards.*—Concentration of working standard depends on expected vitamin K_1 concentration. Dilute ≥1 mL stock solution to 25 mL with hexane to give working standard containing ca 0.01 mg vitamin K_1/mL for use with powdered and ready-to-feed formulas. Samples with high vitamin K_1 concentration, such as concentrates, may require working standard containing ca 0.02 mg vitamin K_1/mL hexane.

Sample Preparation

Reconstitute powdered formulas with 50 mL water and dilute 50 mL samples of concentrates with 50 mL water. Sample ready-to-feed formulas directly. Sample aliquots, generally 50–100 mL, must contain 0.01–0.02 mg vitamin K_1 and ≤4 g fat. Transfer aliquot to 1 L round-bottom flask. Add 200 mL pH 7.7 buffer and 2.5

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Table 1. Statistical treatment of results of HPLC determination of vitamin K_1 in infant formulas

Declared value, μg	Anal. results, μg	Mean	Std dev.	Dev. duplicate anal. results, %
66	114 111	112.5	1.5	1.33
55	89 84	86.5	2.5	2.89
150	310 270	290	2.0	6.71
50	101 105	103	2.0	1.94
24	52 48	50	2.0	4.0
100	140 150	145	0.5	4.53

g lipase powder (Sigma Chemical Co.). Incubate at 37°C for 1.5 h while vigorously stirring with magnetic stirrer. Immediately transfer hydrolysate to 1 L separatory funnel containing 10 mL 10N NaOH and add 200 mL 95% ethanol. Immediately extract with three 200 mL portions of *n*-pentane (glass-distilled). Combine *n*-pentane extracts and, without delay, wash combined extracts with water until neutral to phenolphthalein. Dry organic phase over anhydrous sodium sulfate and evaporate to dryness at 40°C under reduced pressure using rotary evaporator. Dissolve residue in 10 mL hexane and quantitatively transfer to calibrated 15 mL conical centrifuge tube. Evaporate extract to 1 mL.

Chromatography

Sample preparation and chromatography should be performed on same day. Delay between steps results in low recoveries.

Use operating conditions given in *Reagents and Apparatus* (**d**). Inject 10 μL aliquots of sample extracts and working standard solution into instrument.

Results and Discussion

We initially assayed vitamin K_1 in infant formula extracts prepared using the AOAC method (6) for oil-soluble vitamins. The method involves sample hydrolysis with potassium hydroxide followed by alumina column cleanup. For the liquid chromatographic separation, 90% methanol–10% water was used as the mobile phase with the Waters μBondapak C_{18} column. Poor resolution was obtained and vitamin K_1 appeared to co-elute with lipids. Substituting

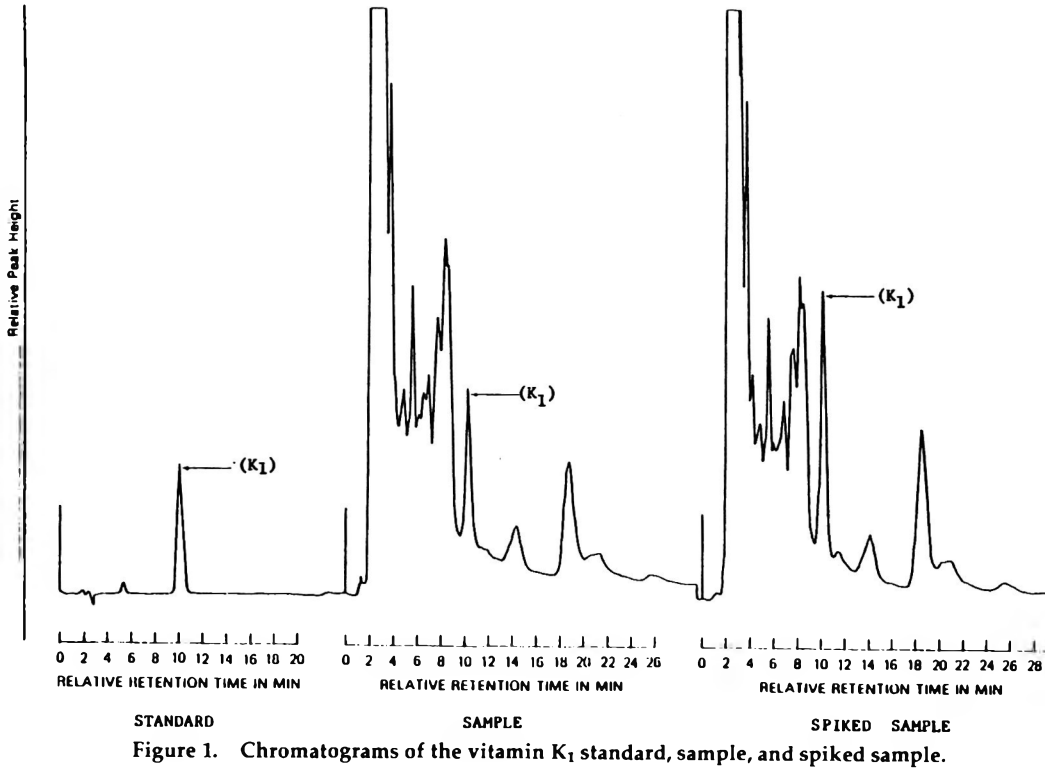

Figure 1. Chromatograms of the vitamin K_1 standard, sample, and spiked sample.

the Zorbax column used by Barnett et al. (5) did not improve resolution.

The Barnett et al. method of sample extraction and chromatography was investigated using a 313 nm detector. Acetonitrile–tetrahydrofuran–ethyl acetate was the first solvent system tried. The analyte of interest co-eluted with other lipids and was difficult to identify. There was some separation of vitamin K_1 but resolution was not acceptable. Barnett visited our laboratory while development of the method was in progress and was told that the separation difficulty was due to the column packing, which he was trying to resolve with the manufacturer (Barnett, S., 1982, personal communication). To gain better chromatographic resolution, a solvent system of acetonitrile–tetrahydrofuran–water was tried using the Waters μBondapack C_{18} column and a 254 nm detector. Separation was poor and required too long a time. The polarity of the solvent system was adjusted by changing the tetrahydrofuran concentration. The vitamin K_1 peak was still not distinct and well resolved at various concentrations of tetrahydrofuran. Using the same solvent system, 2 different columns, SI-60 and Zorbax ODS, were used in series and the sample extract was dissolved in acetonitrile–ethyl acetate (50 + 50) as mentioned by Barnett et al. (5) rather than hexane, but the resolution was still poor and some negative peaks were detected. Finally, a μBondapak C_{18} column and a mobile phase of acetonitrile–methanol–tetrahydrofuran–water (39 + 39 + 16 + 6) with detection at 254 nm achieved separation with good resolution, and the vitamin K_1 peak was sharp and acceptable.

The time of enzymolysis was increased from the 1 h suggested by Barnett et al. to 1.5 h, and methanol was added to the mobile phase. The additional 30 min incubation was necessary to eliminate background interferences that showed up under our modified chromatographic conditions. Good chromatographic resolution was achieved using acetonitrile–methanol–tetrahydrofuran–water (39 + 39 + 16 + 6). The analytes were completely separated in 1 h. Vitamin K_1 eluted at a retention time of 10 min. Figure 1 shows chromatograms of the standard, sample, and spiked sample. Relative SD values of duplicate assays on 6 samples ranged from 1.33 to 6.71% (Table 1). Recoveries of reference standards prepared the same way as samples were 97.5–98.5%. Precision of the modified analytical method is acceptable, as shown by the low standard deviations of duplicate analyses (Table 1).

The use of an internal standard would have

Table 2. Results of HPLC determination of vitamin K_1 in infant formulas

Formula type	Declared, μg/qt	Found, μg/qt	% of declared	Recovery					
				Sample size, mL	Found, μg	Added, μg	Sample + spike, μg	Recd, μg	Rec., %
Concentrate	50	138	275	50	7.3	9.5	15.29	8.0	84
Concentrate	50	118	236	100	12.49	9.5	22.32	9.8	103
Powder	100	122	122	14[a]	14.35	19	31.87	17.5	92
Powder	100	146	146	19[a]	19.95	19	39.50	19.50	103
Powder	100	166	166						
Ready-to-feed	100	157	157	100	16.59	19	32.66	16.07	85
Ready-to-feed	74	133	179						
Ready-to-feed	55	68	124						
Ready-to-feed	66	114	173						
Ready-to-feed	100	137	137	150	21.72	19	38.05	16.30	86

[a] Sample size in g.

improved accuracy and precision but the author did not have any on hand that could be used. Since time was short and compliance with the Infant Formula Act had to be met, the authors instead ran several samples with the same code in duplicate plus a spike each day for more than 10 days. The results obtained from day to day were reproducible and in good agreement. The results from a ready-to-feed, a concentrate, and a powder sample agreed with the manufacturers' results of the same code analyzed by their laboratory.

Ten infant formulas were analyzed for vitamin K_1, including ready-to-feed, concentrated, and powdered samples. Samples were randomly selected from regulatory samples. The results of the analyses obtained using the modified HPLC method are summarized in Table 2. Recovery of vitamin K_1 added to infant formula samples ranged from 84 to 103%. The variations between the declared vitamin K_1 concentrations and those obtained by analyses are an order of magnitude greater than the accuracy of the analytical method, as indicated by the recovery efficiencies of spiked samples.

To obtain high recoveries, the sample extract was prepared and injected on the same day. To maintain good resolution, the μBondapak C_{18} column was routinely cleaned and regenerated after 2 days' use by sequentially passing 75 mL methanol, 100 mL water, 100 mL methanol, 25 mL tetrahydrofuran, 75 mL chloroform, 25 mL tetrahydrofuran, and 100 mL methanol through the column.

Conclusion

A reliable and reproducible HPLC method for the determination of vitamin K_1 is presented. The method is suitable for survey and regulatory analyses.

Acknowledgments

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FOOD ADDITIVES

Determination of Styrene Migration from Food-Contact Polymers into Margarine, Using Azeotropic Distillation and Headspace Gas Chromatography

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Migration studies were conducted to determine the quantity of styrene that migrates from polymers into fatty foods, specifically margarine. Azeotropic distillation was used to isolate styrene from the margarine. Headspace gas chromatography with a Chromosorb 104 column and a flame ionization detector was used for quantitation. The quantitation limit for the method was about 25 ppb (wt/wt) styrene in margarine. On the average, greater than 90% of the styrene was recovered. Several commercial margarines were examined. The method and results of the migration studies are presented. There was no detectable migration of styrene into margarine.

Several adverse health effects are attributed to styrene. Humans experience eye, nose, throat, and skin irritation when exposed to the vapors (1, 2). Styrene has a toxic effect on the liver (3–6), acts as a depressant on the central nervous system, and causes neurological impairment (1, 2, 7–9). An increase in the frequency of chromosomal aberrations has been observed in the lymphocytes of human subjects occupationally exposed to styrene (10–13).

Styrene is the second most widely used monomer in food-contact packaging polymers. Excluding housewares, 336 million pounds of polystyrene were used in 1978, primarily in packaging for fresh red meat and poultry, cottage cheese, fresh fruits and vegetables, cookies, and delicatessen food (14).

Previous work conducted by this laboratory indicated that styrene levels of 60–2250 ppm were present in polystyrene food packaging (15). The monomer migrated into food-simulating solvents from various styrene-containing beverage cups. In studies simulating filling and storage at room temperature, the average values for styrene migrating into 8% ethanol, μg/sq. cm of container, were 0.036 (27 ppb) for foam cups, 0.064 (52 ppb) for impact polystyrene containers, and 0.210 (151 ppb) for crystal polystyrene glasses. Using water, coffee, and tea, an average of 0.0078 μg/sq. cm (6.3 ppb) styrene migrated from foam cups under conditions simulating hot filling or pasteurization above 65.6°C (16).

The migration levels of styrene in substances with a high oil content would be expected to be higher than those mentioned above. Previous studies have shown that the monomer migrates into corn oil about 6–10 times faster than into water (17, 18). In 1978, approximately 131 million pounds of styrene-containing polymers were used in packaging meat, poultry, and fish, 93 million pounds in packaging dairy products, and 5 million pounds in packaging edible fats and oils, specifically margarine (14). Studies were undertaken to determine the migration of styrene monomer from packaging into fatty foods. For this purpose, commercial samples of margarine packaged in styrene-containing polymers were examined.

Various methods have been used to determine the levels of styrene in foods. Withey and Collins (19) developed a headspace gas chromatographic (GC) method of analysis; the determination limit for styrene monomer in margarine was 1 ppm. A headspace technique was also used by Davies and Dunn (20) to analyze a synthetic fat mixture; styrene levels in dairy products, simple aqueous foods, and chocolate spread were determined with a heptane extraction method followed by GC. Brun et al. (21) used a hexane extraction method to analyze wine and a methanol extraction/distillation method to analyze yogurt. Using GC for quantitation, the determination limit for styrene was 10 ppb. Steam distillation has been employed in conjunction with GC to determine the concentration of styrene monomer in a variety of foods (22, 23). A determination limit of about 10 ppb was obtained by Gawell and Larsson (24), using azeotropic distillation with methanol followed by reverse phase liquid chromatography. The recovery of styrene from cooking oil was 55.5% at a concentration of 360 ppb.

Because of the high detection limit for the

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headspace GC method and the high solubility of margarine in heptane and hexane, distillation was chosen as the technique to separate styrene from the bulk of the margarine sample. Azeotropic distillation with methanol yielded the highest recoveries. A Bantam-ware distillation head was used; the use of a reservoir ahead of the collection vessel allowed for the concentration of styrene in the distillate and resulted in higher recoveries. To achieve a low limit of measurement, headspace GC with a flame ionization detector was used for quantitation of the styrene in an aliquot of the distillate. The method and results of the migration studies are presented.

Experimental

Reagents

(a) *Methanol.*—Distilled-in-glass grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). Analyze by headspace sampling technique to assure absence of interferences at retention time of styrene.

(b) *Styrene.*—99%, stabilized with 10–15 ppm *p-tert*-butylcatechol (Aldrich Chemical Co., Milwaukee, WI 53233, No. S497-2). Store in freezer in amber bottle. Confirm purity by GC using flame ionization detector. In spite of presence of inhibitor, some polymerization may take place. Remove polymer by filtering through Whatman No. 40 paper.

(c) *Water.*—Distilled. Test under conditions of analysis to assure absence of substances with approximate retention time of styrene.

Apparatus

See ref. 16 for description of screw-cap glass bottles, syringes, headspace sample vials, and forced air oven.

(a) *Distillation head.*—Bantam-ware No. K287600 (Kontes Glass Co., Vineland, NJ 08360). (The glassware used was specially prepared following the design of the Bantam-ware distillation head without including the tubing for vacuum attachment. The apparatus is shown in Figure 1.) Using the commercial apparatus, keep stopcocks open leading to point of vacuum attachment. Cool cold finger with water that is passed through 26 ft (7.9 m) of 1/4 in. (0.6 cm) od copper tubing coiled in ice bath.

(b) *Collection vessel.*—Glass-stopper 15 mL centrifuge tube graduated in 0.1 mL increments. To cool, place tube in mixture of dry ice and ethanol.

(c) *Variable transformer.*—Powerstat type 3PN116B with 0–140 V output (The Superior Electric Co., Bristol, CT 06010).

(d) *Gas chromatograph.*—Hewlett-Packard Model 5840A equipped with terminal, 4-port valve, and flame ionization detector (Hewlett-Packard, Avondale, PA 19311). Operating conditions: injection port 210°C, column and valve 185°C, detector 230°C; nitrogen carrier flow 30 mL/min (as measured by flow sensor installed in instrument), hydrogen 25 mL/min, air 250 mL/min; chart speed 1 cm/min.

(e) *GC column.*—Coiled 6 ft (1.8 m) × 1/8 in. (0.3 cm) od stainless steel, packed with 60–80 mesh Chromosorb 104. Condition column overnight at 225°C or until acceptable noise level is obtained at lowest attenuation setting of gas chromatograph. To allow for venting, insert 4-port valve after first 2 ft (61 cm) of column. (Only 3 ports are used; cap fourth.)

Preparation of Standards

Stock standard solution.—Accurately weigh 1 oz narrow-mouth bottle, screw cap, and septum. Pipet ca 25 mL methanol into bottle, cap bottle, and reweigh. Using 50 μL liquid syringe, add 45–50 μL styrene to methanol by quickly uncapping bottle and injecting monomer. (This procedure is used to avoid puncturing septum, which would shorten lifetime of standard.) Immediately recap bottle, and thoroughly mix solution by shaking. Reweigh bottle, and calculate styrene concentration (ca 2000 ppm). Stock solution can be used at least 1 month if stored in freezer.

Working standard solutions.—Dilute stock standard solution with methanol to prepare working standard solutions of ca 700, 200, and 70 ppm styrene. Pipet calculated amount of stock standard solution into 1 oz narrow-mouth bottle containing known volume of methanol so that desired concentration of styrene is achieved. Quickly cap bottle and thoroughly mix solution by shaking. Store working standard solutions in freezer to ensure stability of ca 1 month.

Headspace standard solution.—Prepare in range of 25–150 ppb styrene from styrene working standard solutions. Pipet 15 mL water–methanol (90 + 10) into 23 mL sample vial. Add 5 g anhydrous Na_2SO_4 and stopper. Shake thoroughly to dissolve bulk of salt. Unstopper vial, and add predetermined volume of desired styrene working standard solution, using 10 μL liquid syringe. Restopper, cap, and shake vial. Prepare solution fresh daily.

Distillation

Accurately weigh ca 10 g margarine into 100 mL round-bottom flask. Add 40 mL water, 5 mL

methanol, and several carborundum boiling stones. Attach flask to Bantam-ware type distillation head (Figure 1). Place heating mantle around sample flask and set attached variable transformer at 80. To limit heat loss, wrap exposed portion of flask and neck and side arm of distillation head with aluminum foil. Initially direct tip of cold finger toward sample flask. After liquid has started accumulating in collection reservoir, let sample reflux for additional 5 min and then rotate cold finger to direct distillate toward reservoir. Continue refluxing 30 min. Slowly open stopcock on distilling head and collect distillate in receiver tube at rate of 1 drop/8–10 drops falling from tip of cold finger into reservoir (ca 10:1 reflux ratio). After ca 3 mL distillate has accumulated, stop distillation. Measure and record volume collected. Immediately pipet 1.5 mL aliquot into 23 mL glass headspace vial containing 13.5 mL water and 5 g Na_2SO_4 which have been shaken to dissolve bulk of salt. Cap vial and shake.

Headspace Sampling Technique

Place sealed headspace vials containing sample or standard in 60°C oven and let equilibrate. After 30 min, shake vials 1 min to help break up salt. Sample headspace after 1 h, using gas syringe also heated to 60°C. Insert syringe needle through vial septum. Pump plunger several times to wet walls of syringe with sample vapors. Draw 1 mL headspace with syringe and let vapors equilibrate between vial and syringe for 1 min in oven. Close syringe valve, withdraw needle from vial, and quickly transfer sample to gas chromatograph. After inserting syringe needle into injector, open valve and inject headspace sample. Program gas chromatograph terminal to open vent valve for 2 min immediately following start of run. To prevent salt in sample solutions from clogging needle, rinse syringe between injections with water followed by methanol.

Recovery Study

Margarine samples packaged in styrene-free containers were obtained for the determination of the amount of styrene recovered, using the method developed. The margarine was stored under refrigeration until used. The samples were then allowed to come to room temperature. The margarine was removed from the container, placed in a 400 mL beaker, and mixed well using a spatula; 10 g was then accurately weighed into a 100 mL round-bottom flask. All samples were weighed on the same day. Flasks containing those samples not immediately analyzed were stoppered and refrigerated. After the addition of water and methanol, the margarine samples were spiked with styrene working standards. The resulting mixtures were analyzed for styrene by the azeotropic distillation method and headspace GC. By comparing the GC response of the sample to that of a standard, the quantity of styrene in nanograms was determined and the percent recovery was calculated. The spiked samples had concentrations of ca 25, 75, and 300 ppb on a margarine basis. Four determinations were conducted at each level, with two 1.5 mL aliquots being analyzed from each distillation. A margarine blank was also run on each day of analysis.

Commercial Product Analyses

Several commercial products were obtained to study the migration of sytrene from polymers into margarine. The samples were analyzed soon after purchase and before the product expiration date. The margarine was stored and prepared as for the recovery studies. Ten g samples were then accurately weighed. The azeotropic distillation method and headspace GC were used for the analysis. Calibration curves over the concentration range of 25–150 ppb styrene were prepared daily to quantitate any styrene that had migrated. Three determinations were conducted on each commercial sample. Two 1.5 mL aliquots from each distillation were taken for GC analysis.

Infrared (IR) spectra were obtained to qualitatively identify the polymer from which the margarine tubs were prepared and to confirm the presence of styrene. One-half-inch square (3.2 sq. cm) pieces were cut from the containers. Each section was placed between 2 sheets of thin aluminum foil and pressed into a thin (1–2 mil) film with a Rucker PHI Model SP-215C heated bench press operated at 193°C and 30 000 lb ram force pressure. A Perkin-Elmer Model 735B double-beam grating IR spectrophotometer was used to obtain the spectrum from each film. The qualitative composition of each polymer sample was determined from comparisons with standard spectra.

Results and Discussion

Method Development

For the determination of styrene in margarine, a review of the literature indicated that the lowest determination limits and highest recoveries would probably be obtained using azeo-

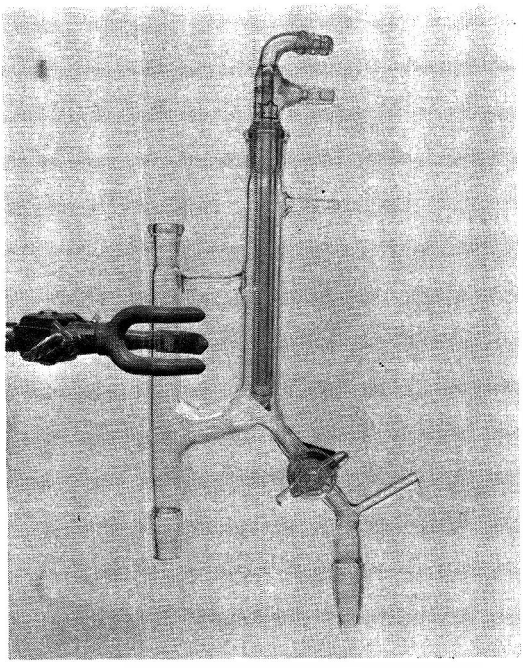

Figure 1. Distillation head with cold finger (for emphasis, tip of cold finger has been darkened).

tropic distillation followed by headspace GC. Initially, steam distillation was tried as the procedure to separate the indirect food additive. Both in situ and external generation of steam was attempted, with recovery levels of only 20%. By adding an 8% ethanol solution to the margarine sample and distilling, 50–60% of the styrene was recovered. In an attempt to improve the results, several other solvents that form azeotropes with styrene were used. *n*-Propyl alcohol was found to contain a large interfering component even after redistillation. Using a 10% solution of isopropyl alcohol as the distilling agent resulted in approximately 40% recovery of the styrene from the margarine.

Distillation of the margarine samples with aqueous solutions of methanol yielded the highest recovery levels. Using the conventional distillation apparatus, about 60% of the added styrene was recovered. When 100% methanol was the distilling agent, only 30% recovery was obtained. Larger amounts of styrene were recovered from the margarine by adding increasing volumes of water. The rate of distillation decreased, however, as more water was added. With 10 g margarine and 5 mL methanol, the use of 40 mL water was a good compromise between recovery and distillation rate.

Because the recovery levels were somewhat less than optimum, a Bantam-ware style distillation apparatus was tested (Figure 1). With a reservoir between the cold finger and the exit valve, styrene could be concentrated in the distillate before collection, thus potentially increasing recovery levels. Lengthening the time that the sample refluxed increased the amounts of styrene recovered. A reflux time of 30 min yielded recoveries of greater than 80%, as desired.

Several steps were taken to improve the efficiency of the distillation. The exposed portion of the sample flask and the neck and side arm of the distillation head were wrapped with aluminum foil to limit heat loss. Thus, the reflux rate and the overall speed of the distillation procedure were increased. Because the temperature was variable, the tap water used to cool the cold finger was passed through copper tubing coiled in an ice bath. The efficiency of condensation improved and the potential loss of styrene decreased. Upon sitting at room temperature, the collected distillate lost styrene. When the collection tube was placed in ethanol and dry ice, this loss was eliminated.

Initially, for GC analysis 1 mL distillate was added to 9 mL water in a 23 mL headspace vial. When the final volume of liquid in the vial was increased to 15 mL, i.e., 1.5 mL distillate and 13.5 mL water, a larger styrene peak resulted. Because the volume taken for analysis remained the same, the actual percentage of headspace injected into the gas chromatograph was increased.

Compounds of high ionic strength have been used to enhance the vapor concentrations of volatile organic components in headspace GC (25). Various amounts of sodium sulfate were added to the sample vials to increase the sensitivity of the analysis. Increasing the amount of salt added from 1 to 5 g resulted in increases in the peak heights of styrene, with a 3- to 4-fold enhancement. The addition of 5 g salt yielded a saturated solution even after 1 h equilibration in the oven. At lower salt concentrations, inconsistent peak heights were obtained. For all further work, 5 g Na_2SO_4 was added to the headspace vials before analysis.

As previously found (16), the time needed to adequately equilibrate the headspace samples in the oven was 60 min. Increasing the time to 90 min resulted in only a slight increase in the heights obtained for the styrene peak.

The column used previously for headspace GC of styrene was 6% Carbowax 20M on Chromosorb 101 (16). In testing this column initially for the

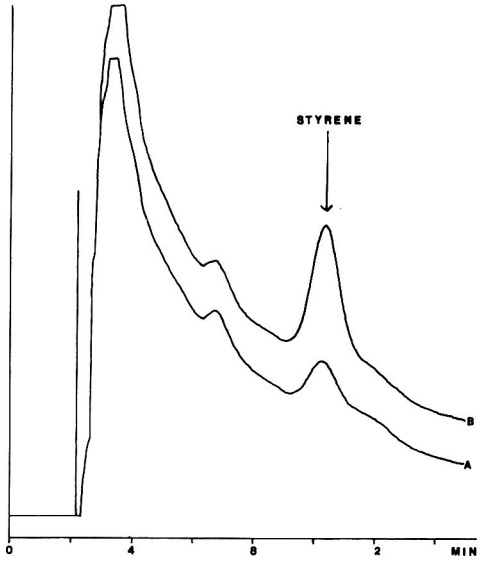

Figure 2. Gas chromatograms of 1 mL headspace injections from A, 25.2 ppb styrene standard; and B, 75.5 ppb styrene standard. Heights of styrene peaks are ca 7% FSD in A and 24% FSD in B.

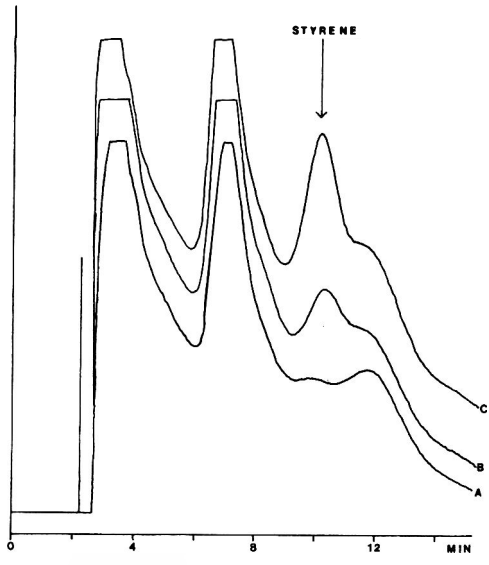

Figure 3. Gas chromatograms of 1 mL headspace injections from A, margarine blank; B, recovery study at 24.9 ppb level of styrene; and C, recovery study at 75.1 ppb level of styrene. In B, height of styrene peak is ca 8% FSD and in C, 23% FSD.

quantitation of styrene from margarine, the indirect food additive eluted on the leading edge of a larger peak and a small interfering peak was present at the same retention time. An uncoated Chromosorb 101 column was tried. Although styrene was better separated from the other peaks present, some overlapping remained, along with an increase in the background slope and a decrease in sensitivity. In an attempt to improve the chromatography, several other columns were tested. A column packed with Chromosorb 104 yielded acceptable chromatography. Coating the packing with 3% Carbowax 20M improved the peak shape somewhat but resulted in an overlap of styrene with a later eluting peak. Thus, a column packed with uncoated Chromosorb 104 was used to quantitate styrene from margarine.

The methanol present in the headspace tailed when chromatographed, causing a sloping background for the styrene peak. As found previously (16), by venting the column immediately after sample injection, this background could be reduced. (The column was vented 2 ft (61 cm) from the end nearest injection.) A vent time of 2 min decreased the slope by one-half and ensured that no styrene was lost.

Margarines prepared from several different kinds of oils—vegetable, sunflower, and corn—were examined to assure the absence of interfering peaks when the above GC procedure was used. In general, no peaks were seen at the retention time of styrene. Margarine samples that were left open for 1-2 weeks developed a greasy film on the surface. Several of the peaks in the chromatogram of margarine increased and styrene eluted on the tail of another peak.

Using the headspace GC procedure developed, the retention time of styrene was approximately 10 min. Defining the determination limit as that concentration yielding a peak height of 10% full-scale deflection (FSD), a styrene level of about 25 ppb could be determined. Chromatograms of styrene at this level and at about 75 ppb are shown in Figure 2. The linear range of the method was determined from analysis of styrene standard solutions. The headspace calibration curve was linear from at least 300 ppb down to the quantitation limit.

Recovery studies were conducted using margarine packaged in a styrene-free container. (The margarine was made with sunflower oil.) Sample chromatograms are shown in Figure 3. A very small interference was present at the retention time of styrene. The height of this peak was subtracted from that of styrene before calculation of the percent recovery. Average recovery for 25.0, 24.9, 25.0, and 25.6 ppb added

styrene was 102 ± 0.5%; average recovery of 75.1, 75.5, 75.1, and 74.7 ppb added styrene was 91 ± 4.2%; and average recovery of 296, 296, 294, and 296 ppb added styrene was 92 ± 3.4%.

Migration Studies

To study the migration of styrene from polymers into margarine, 7 margarine samples were purchased from local area stores. (Four of the products were made from 100% corn oil and 3 from vegetable oils.) IR spectra of films prepared from the containers confirmed that the polymer contained styrene. A comparison with standard spectra indicated that the materials were similar to a styrene-methyl methacrylate copolymer or a styrene-butyl acrylate copolymer (26).

The levels of residual styrene in packaging materials were determined using a method developed previously (15), with slight modifications. The liquid chromatographic column used was 4.6 mm id × 250 mm packed with 5 μm Altex Ultrasphere Octyl (Rainin Instrument Co., Inc., Woburn, MA 01801, No. 256-08). To resolve the styrene peak from those of the interferences, a mobile phase of tetrahydrofuran–water (50 + 50) was used with a flow rate of 0.8 mL/min. Two tubs of each type of margarine, or 4 tubs in all, were examined. Three 1 g portions were taken from each container. The residual levels of styrene were calculated to be 401, 344, 371, and 376 ppm with standard deviations of 2.3, 4.0, 8.5, and 5.0, respectively.

Azeotropic distillation and headspace GC were used to carry out the determinations. There was no evidence of the presence of styrene in any of the margarine samples analyzed. To confirm the adequacy of the chromatography for the determination of styrene, one aliquot of distillate from each margarine sample was spiked at the 50 ppb level. Chromatograms are shown in Figure 4. A peak was noted at a retention time of about 11–12 min. In general, this peak was broad and small. It was not completely resolved from the styrene peak in all cases, but because of its location under the tail, it would not interfere with the peak height measurement and, hence, quantitation of styrene. One of the samples of margarine exhibited a peak with a height of about 50% FSD at a retention time of 11.9 min. A spike of the sample confirmed that styrene could be qualitatively identified if present in this sample. Because of the location of the styrene peak on the leading edge of this larger peak, however, a calculation of styrene concentration would be uncertain.

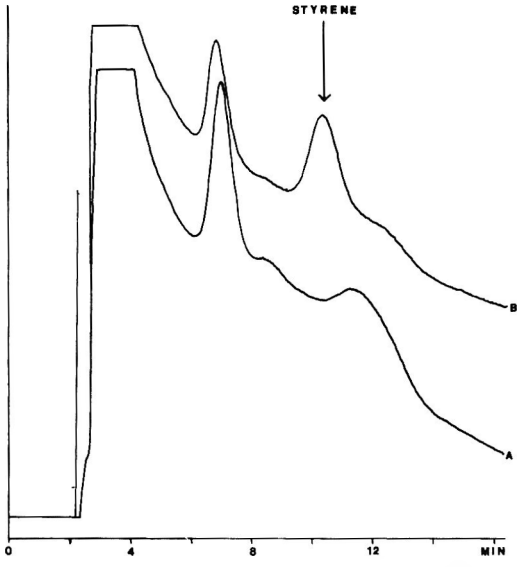

Figure 4. Gas chromatograms of 1 mL headspace injections of aliquots from analysis of margarine from styrene-based tubs, A, unspiked; and B, spiked with styrene at 50.6 ppb level. In B, height of styrene peak is ca 16% FSD.

Previous studies have shown that styrene migrates from polymeric containers into foods. Withey and Collins (19) found that styrene migrated into butter fat cream at levels of 22 ppb after 5 days, 44 ppb after 17 days, and 59 ppb after 24 days. Lower levels of migration occurred in cottage cheese, sour cream (except for one sample which yielded outlying results), yogurt, homogenized milk, yopi (cultured milk), and honey. Studies conducted by Davies (27), using impact polystyrene with residual styrene levels of 500 ppm, showed that the monomer migrated into single cream (19.5% fat) at concentrations of 20 ppb after 1 day, 107 ppb after 7 days, and 170 ppb after 21 days, and into double cream (53% fat) at levels of 20 ppb after 1 day, 150 ppb after 7 days, and 270 ppb after 21 days.

A few studies have been conducted on the migration of styrene into margarine. Withey and Collins (19) did not find any styrene migration from commercial polystyrene containers, using a method having a determination limit of 1 ppm. In crystal polystyrene with residual levels of 800 ppm styrene, levels of 31 ppb styrene were found in margarine after 90 days at 4°C (28). A comparison with the study presented here cannot be made because of the difference in the polystyrene material examined in

the above work and the copolymer material examined in this work.

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TOBACCO

Application of Tenax Trapping to Analysis of Gas Phase Organic Compounds in Ultra-Low Tar Cigarette Smoke

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A method well suited to the analysis of gas phase organic compounds in ultra-low tar delivery cigarette smoke has been developed. The cigarette is smoked directly through a filter and a Tenax trap arranged in series. The components collected on Tenax are analyzed by thermal-desorption programmed-temperature gas chromatography. Components are then quantitated by the use of external standards. The smoke from high tar delivery cigarettes also can be analyzed by thermal desorption gas chromatography of portions of well mixed solid dilutions of the trapping Tenax. Deliveries of 34 gas phase components were determined for 4 ultra-low tar cigarettes having tar deliveries ranging from <0.01 to 3 mg tar/cigarette, and 24 components for 5 cigarettes delivering 7 to 45 mg tar/cigarette.

The analysis of gas phase organic compounds in smoke from ultra-low tar cigarettes where the amount of sample is extremely limited requires an efficient and sensitive analytical method. Current methods developed with relatively high tar delivery cigarettes usually employ cryothermal trapping of the gas phase components (1–4); however, single puffs or a collection of puffs in a large syringe have been directed to a column in a gas chromatograph (GC) through the gas sampling valve (5). Other methods involve condensing whole smoke, followed by fractionation (6) or distillation of volatile compounds (7, 8) to the GC column. Also, devices have been developed to generate an "average puff" (9) of smoke, the gas phase of which is delivered directly to the GC column (10). Although all of these methods have proven applicable to the analysis of the gas phase of relatively high tar cigarettes, they all have drawbacks when used with capillary column gas chromatography and ultra-low tar (<5 mg/cigarette) cigarettes. Sample can be lost during transfer because of the use of metal sampling valves. Also, the capillary column may become contaminated with particulate matter when whole smoke is sampled. The methods also are applicable mainly to the higher tar cigarette products, and may lack sufficient sensitivity for analysis of recently introduced ultra-low tar cigarettes.

Zeldes and Horton (11) used a different method employing Tenax, an adsorbent resin, to trap the cigarette smoke gas phase. Fractional portions of the cigarette smoke gas phase from each puff were sampled in a loop and were transferred to a Tenax trap using an inert gas sweep. They subsequently thermally desorbed (12) low boiling constituents such as acetaldehyde and acrolein for determination using packed column gas chromatography. Although the collection and analysis of the gas phase is more direct, the fraction of the puff taken is presumed to be representative of the entire puff.

This paper describes an improved method of gas phase collection and analysis in which the cigarette is smoked directly through a Cambridge filter and Tenax trap. The gas phase organic compounds collected on the Tenax resin are determined by thermal-desorption capillary column gas chromatography. This method is particularly applicable to the determination of gas phase constituents from ultra-low tar cigarettes, but can be applied to higher tar-delivery cigarette products also.

METHOD

Materials

(a) *Cigarettes.*—(Cigarettes used in this study (Table 1) were commercial varieties obtained from domestic and foreign markets, except the 1R1 Kentucky Reference cigarette was obtained from the University of Kentucky.) Seal cigarettes in plastic bags and store at −20°C. Condition ≥48 h at standard conditions (13) of 60 ± 2% relative humidity and 24 ± 1°C before use. Select specimens for analysis on the basis of weight (usually within ±20 mg of the average)

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Table 1. Cigarette description

Brand	Physical characteristics: Type[a]	Av. wt, g	Av. resistance-to-draw, mm H_2O	Nominal length, mm	Smoking characteristics: Puff No.	Tar delivery,[b] mg/cigarette
High tar:						
A	O,F,HP	1.583	80	80	18	45
B	K,NF,SP	1.123	70	85	11	36
C	O,NF,SP	0.424	76	65	9	26
D	A,F,HP	0.941	118	85	9	16
Low tar:						
E	A,S,F,SP	0.947	132	85	6	7
Ultra-low tar:						
F	A,DF,SP	0.844	89	85	8	3
G	A,DF,HP	0.715	130	85	8	0.2[c]
H	A,DF,HP	0.932	148	85	9	<0.1[c]
I	A,DF,HP	0.817	100	85	7	<0.01[c]

[a] O = oriental commercial brand, F = conventional filter, K = 1R1 Kentucky Reference cigarette, NF = nonfiltered, A = American commercial brand, S = tobacco substitute, DF = air dilution vents in filter rod, HP = hard pack, SP = soft pack.
[b] Measured at Oak Ridge National Laboratory, except as noted.
[c] Advertised values.

and resistance-to-draw (RTD) (normally within ±5 mm water of the average).

(b) *Tenax traps.*—Treat Tenax-GC® (35–60 mesh, Applied Science Laboratories Inc., PO Box 440, State College, PA—referred to throughout this paper simply as Tenax) initially with successive batch extractions (2 each) with equal or greater volumes of water, 50% methanol, methanol, ether, and pentane (all solvents reagent grade or redistilled). After last pentane wash, transfer Tenax to large Soxhlet extractor containing large, home-made stainless-steel cloth thimble for continuous extraction with *n*-pentane (Burdick and Jackson) for 48 h. Blow off residual pentane with nitrogen sweep; then condition Tenax in 1 L stainless steel bomb under helium flowing at 300 mL/min for 1 h at room temperature, followed by temperature programming at 2°/min to 250°C and holding for 24 h. Cool to room temperature under flowing helium and transfer Tenax in clean air box to brown bottle having aluminum foil-lined cap.

Load Tenax traps [heavy-wall Pyrex desorption tubes 9 mm od × 5 mm id × 11 cm long, tapered at one end to fit end of GC (Perkin-Elmer 900) injector port (11, 12)] with 220 ± 15 mg Tenax. Hold in place with solvent-washed and thermally conditioned glass wool.

(c) *GC column.*—Use glass capillary column 66 m long, wall coated with 0.21% UCON 50 HB 660. Prepare by method described in reference 14 except for different etching procedure, below:

Fill 0.25 mm id column at room temperature with 2% (w/v) solution of ammonium bifluoride in methanol. Etch statically by immersing column in 50 ± 1°C water for 1 h, cooling to room temperature, flushing ammonium bifluoride solution with 15 mL methanol, and drying with stream of nitrogen before filling with coating solution (0.21% (w/v) UCON 50 HB 660 in ether). Coat column statically (14). This treatment produces a column somewhat acidic in nature, which restricts components determined to neutral and acidic species. Basic compounds do not chromatograph well.

Procedures

Cigarette smoking and sample collection.—Method of sample collection is illustrated in Figure 1, which shows Tenax trap connected between Cambridge filter and single port smoking machine (4) with silicone rubber sleeves. Set puff volume pulled through filter and Tenax trap by smoking machine at 35 mL, using soap film flowmeter to measure volume drawn. Recondition Tenax trap at 250°C under flowing helium at 2–5 mL/min for 15–20 min and cool under double containment. Reassemble equipment and smoke cigarette (one per analysis, at least 3 analyses per cigarette brand) using standard puff parameters (one 35 mL puff of 2 s duration taken per min) (15) to within 2–5 mm of filter overwrap. Take care to minimize drifting of sidestream smoke back toward air dilution holes in filter during smoking.

Puff flow profile measurements made through

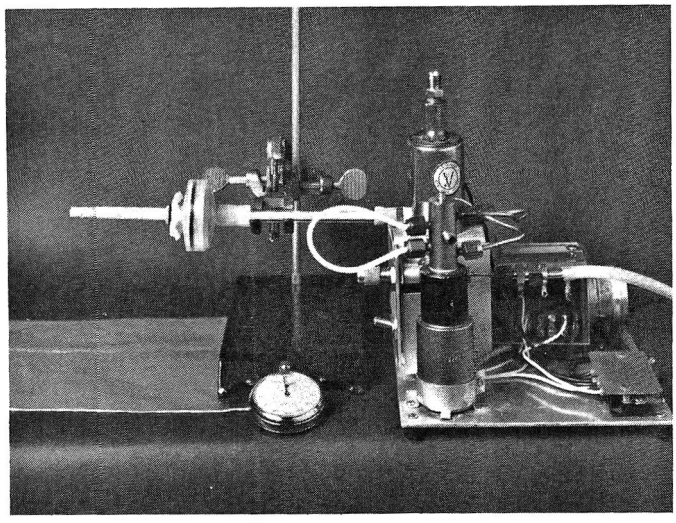

Figure 1. Photograph of cigarette smoke gas phase sampling equipment.

Tenax trap are not different from those taken with trap removed, indicating that presence of Tenax trap does not distort puff profile.

Standards.—Use compounds either present in cigarette smoke or applicable as surrogates for those that are present. Use substances of >97% purity as determined by gas chromatography.

(a) *Standard A.*—Into ca 20 mL redistilled carbon disulfide in 25 mL volumetric flask, accurately pipet with microliter syringe 4–10 μL each of methyl ethyl ketone, benzene, toluene, *n*-nonane, nonene-1, butyl acetate, ethyl benzene, *m*-xylene, *o*-xylene, styrene, and D-limonene. Dilute to 25 mL mark with redistilled carbon disulfide, and mix. From known densities, calculate standard concentrations (ca 0.1–0.3 μg/μL). Transfer to vial sealed with a Teflon-faced septum in an open-faced screw cap and store in a freezer at −20°C. Remove from freezer and warm to room temperature before analysis. Replace punctured septum with fresh septum before returning to storage.

(b) *Solution B.*—Into ca 8 mL redistilled toluene in 10 mL volumetric flask, accurately pipet with microliter syringe 20–50 μL each of 2-methyl butene-1, isoprene, hexene-1, acetone, acrolein, cyclohexene, acetonitrile, methyl ethyl ketone, and benzene. Dilute to 10 mL with redistilled toluene. Mix, transfer, and store as described above. Calculate standard concentrations (ca 2–4 μg/μL).

Analysis.—For cigarettes delivering ≤0.2 mg tar, use entire trap for thermal desorption analysis. For cigarettes delivering >0.2 mg tar, unload Tenax in clean air box into tared vial. Weigh portions of this well mixed sample, or of homogeneous dilution made with a known amount of clean Tenax, for analysis by thermal desorption gas chromatography. Seal the thermal desorption tubes with snug-fitting Teflon end caps to protect Tenax from atmospheric contamination during weighing. This method of sample splitting (diluting with clean Tenax) when analyzing smoke from higher tar delivery cigarettes is simple and practical and gives good reproducibility. We prefer this approach over column inlet splitting because the split ratio is accurately known and is the same for all components reported in this study. There is a risk of sample fractionation if a capillary column inlet splitter is employed with such a wide boiling range mixture (ca 21–175°C).

The thermal desorption analysis method used is essentially the same as described earlier (14) except that only a cryoloop (16) at the front of the GC column is cooled in liquid nitrogen, thereby producing much better peak resolution at the front of the profile. With the injector port temperature at 250°C, liquid nitrogen dewar flask around the cryoloop, and helium carrier gas flowing at >1 mL/min (1 mL/min at room temperature) insert the Tenax trap, richly laden end first, into injector port. In the same operation, add spring which keeps trap snugly fitting, and rapidly recap injector port (use Teflon gasket in injector port cap). After 18 min, remove Tenax trap and recap port. A minute later, remove liquid nitrogen; increase temperature to and hold

Table 2. Breakthrough of gas phase constituents as function of cigarette tar delivery

Constituent	Breakthrough,[a] % Tar delivery, mg/cigarette B(36)	D(16)	F(3)	H(<0.1)
Methyl butene	207	133	10	0
Acetaldehyde + isoprene	174	138	16	0.6
Hexene	45	23	0.4	0
Acetone	97	34	0.7	0
Cyclohexadiene	2	3	0.2	0
Methyl furan	2	3	0.1	0
Benzene	3	0.4	0.5	0
Toluene	0.2	0.8	0.1	0

[a] % Breakthrough

$$= \frac{\text{amount collected on backup trap}}{\text{amount collected on front trap}} \times 100$$

at 0°C for 16 min before programming at 2°/min to 150°C. Components are detected by flame ionization. Detector signal is electronically integrated by a PDP8/e computer which directs a printout of peak areas and retention times.

Components are identified by gas chromatography-mass spectroscopy and verified by comparison of gas chromatography retention times when authentic standards are available. Components are quantitated by the method of external standards, as follows: For most components (benzene and higher boiling substances), add 2 μL Standard Solution A to plug of glass wool in front end of clean Tenax trap while pure air is being pulled through trap at 120 mL/per min for 10 min to transfer standards to Tenax and remove most of the carbon disulfide. For substances more volatile than benzene, add 0.1 μL Standard Solution B directly onto Tenax without pure air flush. Use same thermal desorption GC program as in gas phase analyses to obtain response factors for known compounds. Apply appropriate response factor to integrated peak area for each gas phase component. Multiply weight thus found by dilution factor to obtain amount of each compound delivered by cigarette.

Gas Chromatography-Mass Spectroscopy

Mass spectral verifications of peak identifications were made on a Hewlett-Packard Model 5985 GC/MS system, using same column and chromatographic conditions similar to those of the analytical gas chromatography. Spectra were recorded at 70 eV ionizing voltage in the electron impact mode.

Results and Discussion

The ability of this procedure to analyze the entire collected sample allows, for the first time, sensitivity for the analysis of some ultra-low tar cigarette products. Through simple dilution of the Tenax, gas phase samples collected from higher tar delivery cigarettes also can be analyzed. Profiles of the gas phase from 3 different cigarettes are shown in Figure 2. Profile A is the chromatogram of the gas phase of the nonfiltered 1R1 Kentucky Reference cigarette (brand B, Table 1) which has a tar delivery of 36 mg/cigarette. The Tenax sample was diluted by a factor of 180 to 1. Profile B is the gas phase of an air-dilution filter, ultra-low tar cigarette (brand F, 3 mg tar/cigarette), which required a Tenax dilution factor of 65. Profile C represents the gas phase of the lowest tar delivery cigarette we have tested, also an air-dilution filter cigarette (brand I, <0.01 mg tar/cigarette). No dilution of the Tenax was required in this case.

A comparison of the profiles shows the ability

Table 3. Analytical reproducibility of trapping and analysis procedure as function of cigarette tar delivery

Component	Amount trapped, μg/cigarette (relative standard deviation, %)[a] Tar delivery, mg/cigarette B(36)		D(16)		F(3)		G(0.2)		H(<0.1)		I(<0.01)	
Acetaldehyde + isoprene	(−)[b]	(±23)	(−)[b]	(±21)	92	(±13)	24	(±28)	4.7	(±52)	2.0	(±40)
Acetone	(−)[b]	(±24)	(−)[b]	(±17)	52	(±14)	16	(±35)	0.77	(±38)	0.42	(±74)
Methyl ethyl ketone	131	(±10)	91	(±30)	17	(±23)	4.5	(±18)	0.15	(±57)	0.03	(±46)
Benzene	75	(±17)	57	(±13)	13	(±14)	2.6	(±36)	0.14	(±75)	0.07	(±46)
Toluene	115	(±10)	71	(±28)	12	(±19)	3.4	(±4.0)	0.23	(±24)	0.10	(±22)
m-Xylene	18	(±18)	10	(±21)	1.6	(±30)	0.52	(±18)	0.024	(±50)	0.005	(±50)
Styrene	10	(±16)	6	(±22)	0.61	(±24)	0.12	(±28)	0.005	(±78)	0.002	(±100)
Limonene	34	(±11)	18	(±27)	3.4	(±17)	0.42	(±29)	0.011	(±43)	0.002	(±100)

[a] Based on triplicate analyses (3 separate cigarettes).
[b] Absolute result deleted because of serious breakthrough. All other results are not complicated by breakthrough.

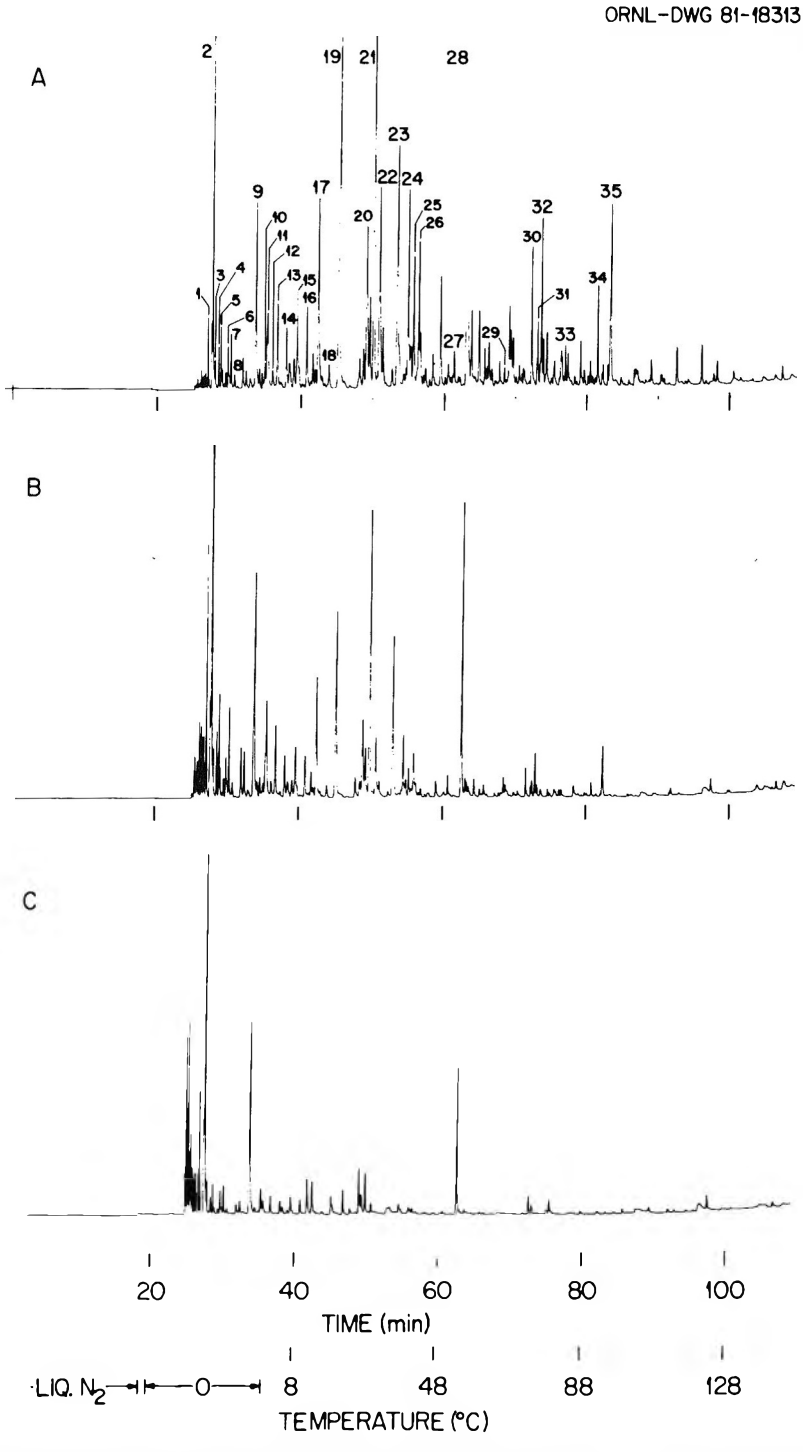

Figure 2. Gas chromatographic profiles of the gas phase from 3 cigarettes: A = high tar delivery 1R1 Kentucky Reference Cigarette (36 mg tar/cigarette), Tenax dilution factor = 180; B = ultra-low tar delivery cigarette (3 mg tar/cigarette), dilution factor = 65; C = ultra-low tar delivery cigarette (<0.01 mg tar/cigarette), no dilution. (See Table 4 for peak identification.)

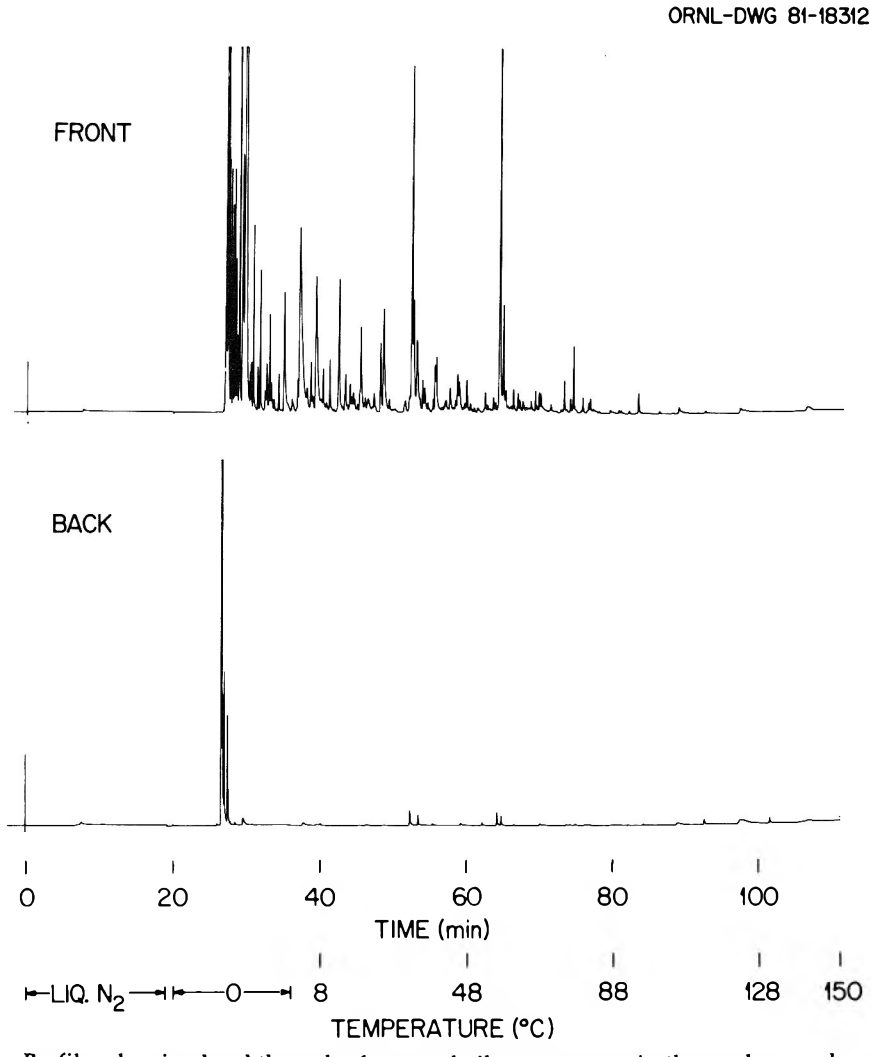

Figure 3. Profiles showing breakthrough of very volatile components in the smoke gas phase from an ultra-low tar (<0.1 mg tar/cigarette) delivery cigarette. Gas phase drawn through 2 Tenax traps in series.

of this procedure to distinguish qualitative differences in the gas phases delivered by the cigarettes. There is practically no limonene (peak 35) or other high boiling components in the gas phase of the ultra-low tar delivery cigarette (profile C). Quantitatively (because of the known dilution factors), peaks of about equal area (e.g., acetone, peak 9) on profiles B and C indicate that there is about 65 times more of that component produced by cigarette B than by cigarette C.

Experiments were performed to determine the efficiency of the Tenax for collecting the gas phase components. Analysis of gas phase components collected on 2 Tenax traps arranged in series produced the results shown in Figure 3. The top profile represents the gas phase of an ultra-low tar delivery cigarette (brand H, <0.1 mg tar/cigarette) smoked through the front trap. The bottom profile shows the components which broke through the front trap and remained on the back-up trap. There is no breakthrough for compounds containing 5 carbon atoms (peaks 1 and 2) and more. Only compounds more volatile than methyl butene (peak 1) are present in significant amounts on the back-up trap. However, it must be noted that many low molecular weight organic compounds (e.g., methane, ethane, propane, and ethylene which are also contained in tobacco smoke) are not retained

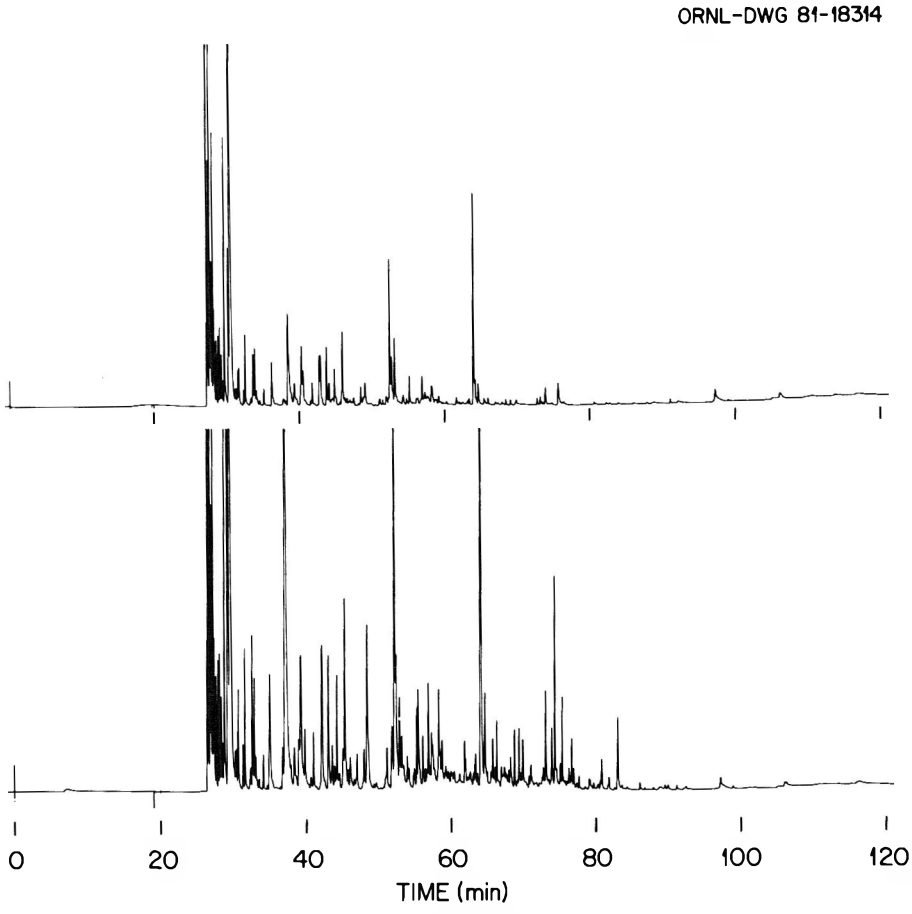

Figure 4. Gas phase profiles of cigarette smoke which drifted away from (top) or occasionally toward (bottom) air dilution holes in filter during smoking.

by Tenax resin and will not be found in either front or backup traps. This represents a limitation of the Tenax trapping method.

There is some breakthrough when higher tar delivery cigarettes are smoked. Table 2 shows gas phase component breakthrough measurements for 4 cigarettes having tar deliveries of 36, 16, 3, and <0.1 mg/cigarette. Breakthrough is nearly undetectable for the gas phase components of the ultra-low tar delivery cigarette smoke and is significant only for compounds of volatility equal to and greater than that of acetone (peak 9, Figure 2) for the high tar delivery cigarettes. Preliminary work suggests that these substances breaking through Tenax resin can be trapped on a carbonaceous material such as Ambersorb XE-340 and subsequently recovered by thermal desorption. Alternatively, if the whole smoke from high tar delivery cigarettes were diluted in the range of 1 to 5%, and known portions of this diluted smoke were sampled through the Tenax, breakthrough would then be negligible.

Reproducibility of the entire sample collection and analysis procedure is an important consideration. Table 3 shows the reproducibility of results for typical gas phase constituents ranging in delivery from >100 μg/cigarette down to the nanogram per cigarette level. The average RSD of the measurements determined for the 1R1 cigarette (36 mg tar) components was ±16%, compared with ±10% for the reproducibility of repeated standards. For the 16 mg tar delivery cigarette, the average RSD was ±22%. For the ultra-low tar cigarettes, the average RSD increased with decreasing tar delivery, and ranged from ±19 to ±60%. It is suspected that cigarette-to-cigarette variability may be an important contributing factor to the RSD, especially for the lowest of the ultra-low tar cigarettes.

The greater variability of the measured component deliveries for the ultra-low tar cigarettes

Table 4. Comparison of results of gas phase analysis of 9 cigarette brands

		Component Delivery,[c] μg/cigarette								
		Tar delivery, mg/cigarette[d]								
Peak[a]	Component[b]	A(45)	B(36)	C(26)	D(16)	E(7)	F(3)	G(0.2[e])	H(<0.1[e])	I(<0.01[e])
1	Methyl butene	—[f]	—	—	—	—	14[g]	4	0.6	0.2
2	Acetaldehyde + isoprene	—	—	—	—	—	107[g]	24	4.7	1.5
4	Cyclopentene	—	—	—	—	—	6	1.7	0.16	0.08
5	Hexene	—	—	—	—	—	5	1.3	0.16	0.05
6	Dimethyl hexane	—	—	—	—	—	4	0.9	0.14	0.05
7	Cyclopentadiene	—	—	—	—	—	7	1.0	0.18	0.06
8	Methyl pentene	—	—	—	—	—	1	0.2	0.05	0.01
9	Acetone	—	—	—	—	—	52	16	0.77	0.40
10	Methyl pentadiene	—	—	—	—	—	1	0.2	0.02	0.01
11	Acrolein	—	—	—	—	—	12	4	0.27	0.09
12	Methyl acetate	—	14	11	10	11	4	0.8	0.02	0.02
13	Methyl pentadiene	13	5	3	3	1	2	0.4	0.03	0.02
14	Cyclohexane	24	19	22	13	5	3	1.1	0.14	0.02
15	Cyclohexadiene	14	16	19	9	6	3	0.7	0.05	0.04
16	Cyclohexadiene	13	12	12	8	4	1.5	0.5	0.06	0.02
17	Methyl furan	86	42	50	23	32	11	1.5	0.23	0.07
18	Methyl cyclopentadiene	6	5	5	4	2	1.5	0.2	0.01	0.02
19	Methyl ethyl ketone	97	131	63	91	63	17	4	0.15	0.03
20	Methyl vinyl ketone	22	44	24	26	29	7	1.6	0.06	0.02
21	Benzene	94	75	72	57	42	13	2.6	0.14	0.05
22	Methyl isopropyl ketone	30	52	38	49	23	5	1.6	0.12	0.03
23	Butanedione	54	60	16	40	50	13	4	0.06	0.01
24	Dimethyl furan	34	27	10	18	10	4	0.3	0.07	0.02
25	Isobutyronitrile	35	46	20	34	11	3	0.9	0.05	0.01
26	Methyl propyl ketone	23	26	13	15	18	4	1.0	0.05	<0.01
27	Nonane	5	4	2	2	0.4	0.1	0.1	<0.01	<0.01
28	Toluene	126	115	71	71	55	12	3.4	0.23	0.10
29	Methyl butyl ketone	1	6	2	2	1	0.3	0.1	0.01	<0.01
30	Ethyl benzene	12	14	11	8	6	1	0.3	0.01	<0.01
31	*p*-Xylene	7	7	8	4	3	0.7	0.2	0.01	<0.01
32	*m*-Xylene	20	18	20	10	4	2	0.5	0.02	<0.01
33	o-Xylene	4	8	10	4	2	0.5	0.1	0.01	<0.005
34	Styrene	8	10	13	6	3	0.6	0.1	<0.01	<0.005
35	Limonene	20	34	33	18	0	3	0.4	0.01	<0.005

[a] Figure 2.
[b] Specific compound isomer listed where both GC retention and mass spectral identifications were made.
[c] Average of determinations on smoke from at least 3 separate cigarettes.
[d] Measured at Oak Ridge National Laboratory, except as noted.
[e] Advertised value.
[f] Result not listed because of excessive breakthrough.
[g] Corrected for known breakthrough.

may not result only from analytical uncertainties and cigarette-to-cigarette variability. A significant portion of the variability may be contributed by the sidestream drifting back toward the cigarette filter and being drawn through the filter air dilution holes and enriching the mainstream smoke during cigarette puffing. Figure 4 shows the gas phase profile of the <0.01 mg tar delivery cigarette (brand I) when the sidestream drifted away (top profile) from the cigarette. The bottom profile is that of the gas phase of the same type of cigarette when the sidestream occasionally drifted back toward the filter. This effect appears to represent a significant potential source of error when experimenting with the ultra-low tar cigarettes if the smoking operation is not conducted carefully.

This new gas phase collection and analysis procedure has been applied to a wide range of commercial cigarette products to demonstrate its applicability. The gas phase deliveries of 34 components ranging from methyl butene to li-

Table 5. Comparison of results for 1R1 Kentucky Reference cigarette with literature values for similar cigarettes

	Delivery, μg/cigarette					
	Whole smoke condensate		Gas phase			
Component	Johnstone et al.[a]	Baggett et al.[b]	Grob[c]	Irby & Harlow[d]	This work[e]	Vickroy[f]
Methyl furan			20	28	42	87
Methyl ethyl ketone			80	86	131	207
Benzene	27–48	23	30		75	104
Dimethyl furan	45–48	58	16		27	135
Methyl propyl ketone			12		26	79
Toluene	46–164	44	80		115	163
Ethyl benzene	7–20		<14		14	
m-Xylene	30–48		16		18	
p-Xylene		19[g]			7	
Styrene		18	10		10	
Limonene	165–200	64	24		34	

[a] Methanol volatile fraction, British and Russian flue-cured tobacco cigarette condensate (6).
[b] American filter blend (19).
[c] Tobacco without additives or filter tip, representative puff or composite (5, 17).
[d] Tobacco without additives or filter tip, 9 puff cigarette (1).
[e] 1R1 Kentucky Reference cigarette, 85 mm length, 11 puffs, 43 mg TPM/cigarette, 36 mg tar/cigarette.
[f] Commercial U.K. flue-cured tobacco cigarettes, 72 mm in length, "average puff" analysis (10).
[g] Sum of *m*- and *p*-xylenes.

monene are shown in Table 4 as a function of cigarette tar delivery for 9 different cigarettes. Tar deliveries varied from 45 mg to 10 μg/cigarette, and gas phase component delivery ranged from more than 100 μg/cigarette to the ng/cigarette level. Results for components which are known to seriously break through or which cannot be reliably corrected for breakthrough have been omitted. Toluene (peak 28) was produced to the extent of 126 μg by the 45 mg tar cigarette (brand A), compared with 0.1 μg by the <0.01 mg tar cigarette (brand I). Analytical results for gas phase aromatic compounds generally correlated with tar delivery, but some components varied considerably with the type of cigarette. For example, the results for the 7 mg tar delivery cigarette show proportionally higher ketone delivery tobacco-substitute and no detectable limonene, in contrast to the other cigarettes.

To evaluate the reliability of our measurements obtained by the Tenax trapping method we compared results for the 1R1 Kentucky Reference cigarette with available literature values for similar cigarettes (Table 5). To our knowledge, no comparative data for low or ultra-low tar cigarettes are available in the literature. Our results for the 1R1 cigarette fall between the gas phase measurements made by Grob (5, 17) and Irby and Harlow (1), and Vickroy (10), and are consistent with the differences in the cigarettes and smoking conditions.

In comparing gas phase analytical results with analyses in which whole smoke was condensed before measurement, only the values from benzene through toluene can be compared directly. This is because *m*- and *p*-xylenes distribute almost equally between particulate phase and gas phase (18) and styrene and limonene distribute more into the particulate phase than into the gas phase (5, 18). Thus, the whole smoke results would be expected to range higher. Except for dimethyl furan, our values are consistently higher than those of Baggett et al. (19), which is to be expected because their cigarette was a domestic filter blend of unspecified but most likely much lower tar delivery. Even the limonene gas phase delivery, when adjusted for retention on the filter pad (18), calculates to approximately 120 μg/cigarette for whole smoke. This result agrees reasonably well with the values obtained by Johnstone et al. (6) using flue-cured tobacco.

These results, obtained with a high tar delivery cigarette, suggest the reliability of our method. We feel that the method of drawing the cigarette smoke gas phase through a Tenax trap followed by thermal desorption gas chromatography is ideally suited for analysis of the gas phase of ultra-low tar delivery cigarettes. It also is applicable to cigarettes with higher tar deliveries and should prove useful for the analysis of semi-volatile smoke components and thermally desorbable particulate matter if applied to whole smoke rather than only the gas phase.

Acknowledgment

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PESTICIDE RESIDUES

Retention Time Data for Organochlorine, Organophosphorus, and Organonitrogen Pesticides on SE-30 Capillary Column and Application of Capillary Gas Chromatography to Pesticide Residue Analysis

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Relative retention time data for 194 pesticides and metabolites are reported for a 15 m SE-30 capillary gas chromatographic column under a single temperature-programmed regime. The reproducibility of retention time and quantitation is discussed and the performance of electron capture and nitrogen-phosphorus thermionic detectors is evaluated in relation to pesticide residue analysis.

Gas-liquid chromatography (GLC) is one of the most important analytical techniques used in pesticide residue analysis. Two advantages are its sensitive and specific detector systems and the ability to separate mixtures of analytes on the column. Until recently, GLC of pesticides has been conducted using packed columns containing a large variety of liquid phases and supports; the wide range of volatilities and specific responses of pesticides has necessitated numerous analytical conditions to chromatograph several classes of pesticides in a single sample. Many pesticides are too polar or contain functional groups that are not amenable to packed column GLC, while others are thermally labile and degrade in the chromatographic system, precluding their determination.

Capillary gas chromatographic (CGC) instrumentation has improved significantly in recent years, particularly with respect to injection systems, microprocessor control of oven temperature, and data handling capability for the large number of peaks produced by the column. CGC column technology has also improved; fused silica CGC columns are flexible and offer ease of manipulation and installation not found with glass capillary columns. These columns are wall-coated with a thin film of liquid phase which produces a large number of theoretical plates so that high resolution of analytes is possible; they provide an inert surface, thus preventing on-column decomposition or adsorption, and allow temperature programming to produce sharp, symmetrical peaks without excessive baseline distortion and retention times that are not prohibitively lengthy.

Capillary gas chromatography has been applied to the analysis of environmental samples for the high resolution separation of isomers and congeners of many priority pollutants (1-3). The technique has also been used to separate individual compounds in several classes of pesticides including the organochlorine (4), organophosphorus (5), and *N*-methylcarbamate (6) insecticides, as well as triazine (7, 8) and phenylurea (8) herbicides.

Several compilations of retention times of pesticides on different packed gas-liquid chromatographic columns have been made (9-13) but no similar qualitative information exists for capillary columns. The object of this investigation was to obtain retention time data for organochlorine, organophosphorus, and organonitrogen pesticides on a SE-30 CGC column, and to evaluate linearity, sensitivity, and specificity of electron capture (ECD) and nitrogen-phosphorus thermionic (NPD) detectors with a capillary gas chromatographic system for the qualitative and quantitative analysis of pesticide residues. In addition to data on response and resolution, we include many personal observations and experiences on the performance of the CGC system that may be of benefit to the reader.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—Hewlett-Packard 5880 with dual capillary injection system, automatic liquid sampler, Level 4 terminal, and ^{63}Ni electron capture and nitrogen-phosphorus thermionic detectors.

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Table 1. Retention time relative to parathion (RRT) of organochlorine, organophosphorus, and organonitrogen pesticides, and some metabolites and derivatives

Compound[a]	PSNX in molecule	RRT[b]	Compound[a]	PSNX in molecule	RRT[b]
Acephate	PSN	0.43	Dichlobenil	NCl_2	0.33
Alachlor	NCl	0.89	Dichlofenthion (VC-13)	PCl_2	0.85
Aldrin	Cl_6	0.98	Dichloran	N_2Cl_2	0.65
Allidochlor	NCl	0.30	3,4-Dichloroaniline	NCl_2	0.39
Ametryn	N_5S	0.89	Dichlorvos	PCl_2	0.27
Aminocarb	N_2	0.70	Dicofol	Cl_5	1.71
Aminocarb phenol	N	0.35	Dieldrin	Cl_6	1.27
AMPA[c] N-TFA-$(OMe)_2$	PNF_3	0.24	Dimethoate	PS_2N	0.66
Atraton	N_5	0.66	Dimethoate oxon	PSN	0.54
Atrazine	N_5Cl	0.68	2,6-Dimethylaniline	N	0.22
Atrazine deethyl	N_5Ci	0.58	Dinitramine	N_4F_3	0.78
Azinphos-methyl	PS_2N_3	1.76	Dinoseb OMe	N_2	0.82
Bendiocarb	N	0.58	Dioxathion	P_2S_4	0.71
α-BHC	Cl_6	0.62	Diphenamid	N	1.03
β-BHC	Cl_6	0.67	Diphenamid demethyl	N	1.00
Binapacryl	N_2	1.38	2,2-Diphenylacetamide	N	0.99
Bromacil	N_2Br	0.93	Diphenylamine	N	0.41
Bromophos	$PSCl_2Br$	1.05	Diquat (reduced)	N_2	0.52
Butylate	NS	0.41	Disulfoton	PS_3	0.76
Bux™(technical)-	N	0.61	Diuron	N_2Cl_2	0.68
bufencarb		0.69	Endosulfan-I	SCl_6	1.19
		0.71	Endosulfan-II	SCl_6	1.34
		0.76	Endosulfan sulfate	SCl_6	1.47
Captafol	$SNCl_4$	1.54	Endrin	Cl_6	1.32
Captan	$SNCl_3$	1.07	EPTC	SN	0.36
Carbaryl	N	0.85	Ethion	P_2S_4	1.43
Carbofuran	N	0.65	Ethion oxon	P_2S_2	1.31
Carbofuran 3-hydroxy	N	0.84	FCR-1272[d]	NCl_2F	2.15
Carbofuran 3-keto	N	0.76			2.17
Carbophenothion	PS_3Cl	1.49			2.18
Carboxin	SN	1.28			2.20
Chlorbromuron	N_2ClBr	1.09	Fenamiphos	PSN	1.25
α-Chlordane	Cl_8	1.21	Fenchlorphos	$PSCl_3$	0.91
γ-Chlordane	Cl_8	1.15	Fenchlorphos oxon	PCl_3	0.82
Oxychlordane	Cl_8	1.10	Fenitrothion	PSN	0.93
Chlorfenvinphos	PCl_3	1.12	Fensulfothion	PS_2	1.38
Chlorothalonil	N_2Cl_4	0.73	Fensulfothion oxon	PS	1.29
Chlorothalonil 4-hydroxy OMe	N_2Cl_3	0.77	Fensulfothion sulfone	PS_2	1.42
Chloroxuron	N_2Cl	1.50	Fenthion	PS_2	0.99
Chlorpyrifos	$PSNCl_3$	1.00	Fenthion oxon	PS	0.89
Chlorpyrifos oxon	$PNCl_3$	0.98	Fenthion sulfone	PS_2	1.39
Chlorpyrifos-methyl	$PSNCl_3$	0.86	Fenthion sulfoxide	PS_2	1.37
CIPC	NCl	0.59	Fenvalerate	NCl	2.37
Coumaphos	PSCl	2.04			2.41
Crufomate	PNCl	1.03			2.48
Cyanazine	N_6Cl	0.97			2.54
Cycloate	SN	0.57	Folpet	$SNCl_3$	1.12
Cypermethrin	NCl_2	2.20	Fonofos	PS_2	0.72
		2.22	Fonofos oxon	PS	0.62
		2.24	Furalaxyl	N	1.14
		2.25	Galben[e]	N	1.50
Cyprazine	N_5Cl	0.83	Glyphosate N-TFA-$(OMe)_3$	PNF_3	0.44
o,p'-DDD	Cl_4	1.30	HCB	Cl_6	0.65
p,p'-DDD	Cl_4	1.40	Heptachlor	Cl_7	0.89
o,p'-DDE	Cl_4	1.19	Heptachlor epoxide	Cl_7	1.09
p,p'-DDE	Cl_4	1.29	Hexazinone	N_4	1.52
DDMU	Cl_3	1.17	Iprodione	N_3Cl_2	1.68
o,p'-DDT	Cl_5	1.43	Isofenphos	PSN	1.13
p,p'-DDT	Cl_5	1.53	Isofenphos oxon	PN	1.03
Demeton-O	PS_2	0.66	Kepone (chlordecone)	Cl_{10}	1.46
Demeton-S	PS_2	0.54	Leptophos	$PSCl_2Br$	1.81
Diallate	$SNCl_2$	0.63	Lindane (γ-BHC)	Cl_6	0.68
		0.65	Linuron	N_2Cl_2	0.95
Diazinon	PSN_2	0.76	Malathion	PS_2	0.98
Diazinon oxon	PN_2	0.73	Malathion oxon	PS	0.87
Dicapthon	PSNCl	1.01	Meclozolyn[f]	N_3Cl_2	1.04

Table 1 cont'd

Compound[a]	PSNX in molecule	RRT[b]
Metalaxyl	N	0.90
Metalaxyl acid	N	1.06
Metalaxyl acid O-PFB	NF_5	1.61
Methamidophos	PSN	0.28
Methidathion	PS_3N_2	1.14
Methiocarb	SN	0.93
Methomyl oxime	SN	0.16
Methoxychlor	Cl_3	1.72
Methoxychlor dehydrochloro	Cl_2	1.51
Metobromuron	N_2Br	0.80
Metolachlor	NCl	0.99
Metribuzin	SN_4	0.82
Mevinphos	P	0.39
Mirex	Cl_{12}	1.83
Molinate	SN	0.47
Monuron	N_2Cl	0.48
Naled	PCl_2Br_2	0.27
Nitrofen	NCl_2	1.33
trans-Nonachlor	Cl_9	1.24
Oxamyl oxime	SN_2	0.47
Oxydemeton-methyl sulfone	PS_2	0.88
Paraquat (reduced)	N_2	0.60
Parathion	PSN	1.00
Parathion oxon	PN	0.89
Parathion-methyl	PSN	0.85
PBB[g]	Br_x	2.28
Pebulate	SN	0.43
Permethrin	Cl_2	2.04
		2.06
Phorate	PS_3	0.62
Phorate oxon	PS_2	0.54
Phorate sulfone	PS_3	0.99
Phorate sulfoxide	PS_3	0.94
Phorate sulfoxide oxon	PS_2	0.82
Phosalone	PS_2NCl	1.80
Phosmet	PS_2N	1.64
Phosphamidon	PNCl	0.75
		0.85
Photomirex	Cl_{11}	1.62
Pirimicarb	N_4	0.81
Pirimicarb demethyl	N_4	0.82
Pirimiphos-ethyl	PSN_3	1.09
Pirimiphos-ethyl oxon	PN_3	1.04
Pirimiphos-ethyl deethyl	PSN_3	1.02
Prometon	N_5	0.68
Prometryn	SN_5	0.90
Propachlor	NCl	0.53
Propazine	N_5Cl	0.69
Propoxur	N	0.53
Pyrethrin	—	1.33
		1.49
Quinomethionate	S_2N_2	1.13
Quintozene	NCl_5	1.71
RE-20615[h]	NCl	1.43
RE-26745[i]	N	1.37
RE-26940[i]	N	1.58
Simazine	N_5Cl	0.66
Simazine deethyl	N_5Cl	0.56
Sulfallate	S_2NCl	0.62
Sulfotep	P_2S_2	0.61
Tebuthiuron	SN_4	0.43
Tecnazene	NCl_4	0.53
TEPP	P_2	0.15
		0.19
Terbacil	N_2Cl	0.74
Terbufos	PS_3	0.72
Terbutryn	SN_5	0.94
Tetrachlorvinphos	PCl_4	1.20
Tetradifon	SCl_4	1.77
Thionazin	PSN_2	1.03
TOCP[k]	P	1.88
Triadimefon	N_3Cl	1.02
Triadimenol	N_3Cl	1.15
Triallate	$SNCl_3$	0.79
Trichlorfon	PCl_3	0.27
Trichloronat	$PSCl_3$	1.05
Trifluralin	N_3F_3	0.64
Vernolate	SN	0.42
Vinclozolin	NCl_2	0.87
Zytron™	$PSNCl_2$	0.97

[a] Compounds by acceptable common name (14–16) or as common derivatives (OMe = methyl ether or ester, N-TFA = *N*-trifluoroacetate, O-PFB = *O*-pentafluorobenzylate, reduced paraquat or diquat (17)).

[b] Major peak is boldface when isomers are present.

[c] Aminomethylphosphonic acid, glyphosate metabolite.

[d] Cyano-(4-fluoro-3-phenoxyphenyl)methyl - 3 -(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (Chemagro Ltd).

[e] Methyl-2-[*N*-phenyl acetyl-*N*-(2,6-dimethylphenyl)-amino]-propanate (Montedison S.p.A.).

[f] Structure not released (BASF).

[g] Polybrominated biphenyl. Major peak from Firemaster BP6; several other smaller peaks.

[h] 2-Chloro-*N*-(2,6-dimethylphenyl)-*N*-tetrahydro - 2 - oxo-3-furanyl) acetamide (Chevron).

[i] 2-Methoxy-*N*-(2,6-dimethylphenyl)-*N*-tetrahydro - 2 - oxo-3-furanyl)acetamide (Chevron).

[j] *N*-(2,6-Dimethylphenyl)-2-methoxy-*N*-tetrahydro - 2 - oxothiophen-3-yl)acetamide (Chevron).

[k] Tri-*o*-cresyl phosphate.

(b) *Capillary column.*—J&W fused silica capillary column, 15 m × 0.247 mm, with 0.25 μm coating of SE-30 (Chromatographic Specialties Ltd, Brockville, Ontario, Canada).

(c) *Chromatographic conditions.*—Temperatures: injector 250°C, detector 300°C. Column oven: programmed for an initial 1 min hold at 90°C, followed by 20°/min to 150°C, and then 5°/min to 250°C with appropriate hold at this temperature to allow elution of late compounds. Gas flows: helium carrier gas 30 cm/s (105 kPa head pressure); 3.0 mL argon–methane (95 + 5)/min make-up gas to ECD; 28 mL helium/min make-up gas, 50 mL air/min, and 3.0 mL hydrogen/min to NPD. The ECD was operated at 2^8 while the NPD was operated at 2^3.

(d) *Injection conditions.*—1–2 μL, either manually or by auto-injection, with capillary inlet system configured in splitless mode with bypass valve open for 0.5–1 min.

(e) *Pesticide standards.*—Stock solutions of 1 mg/mL of analytical reference standards were prepared in appropriate solvents and stored in glass bottles under refrigeration. Suitable

Table 2. Retention time relative to aldrin and parathion of some chlorinated pesticides

Pesticide	Retention time relative to Aldrin[a]	Parathion[b]
Tecnazene	0.54	0.53
α-BHC	0.63	0.62
Dichloran	0.66	0.65
HCB	0.67	0.65
β-BHC	0.69	0.67
Lindane	0.69	0.68
Quintozene	0.72	0.71
Heptachlor	0.90	0.89
Aldrin	1.00	0.98
Parathion (reference)	1.02	1.00
Meclozolyn	1.06	1.04
Captan	1.08	1.07
Heptachlor epoxide	1.11	1.09
Oxychlordane	1.12	1.10
Folpet	1.14	1.12
γ-Chlordane	1.17	1.15
DDMU	1.19	1.17
o,p'-DDE	1.21	1.19
Endosulfan-I	1.21	1.19
α-Chlordane	1.23	1.21
trans-Nonachlor	1.26	1.24
Dieldrin	1.29	1.27
p,p'-DDE	1.31	1.29
o,p'-DDD	1.32	1.30
Endrin	1.35	1.32
Pyrethrum-I	1.36	1.33
Endosulfan-II	1.36	1.34
p,p'-DDD	1.43	1.40
o,p'-DDT	1.45	1.43
Kepone	1.48	1.46
Endosulfan sulfate	1.50	1.47
Pyrethrum-II	1.52	1.49
Methoxychlor-dehydrochloro	1.54	1.51
p,p'-DDT	1.56	1.53
Captafol	1.56	1.54
Photomirex	1.65	1.62
Iprodione	1.71	1.68
Dicofol	1.74	1.71
Methoxychlor	1.75	1.72
Tetradifon	1.80	1.77
Mirex	1.86	1.83
Permethrin I	2.07	2.04
Permethrin II	2.10	2.06
FCR 1272[c] I	2.19	2.15
FCR 1272 II	2.21	2.17
FCR 1272 III	2.22	2.18
FCR 1272 IV	2.23	2.20
Cypermethrin I	2.24	2.20
Cypermethrin II	2.26	2.22
Cypermethrin III	2.27	2.24
Cypermethrin IV	2.28	2.25
PBB[d]	2.32	2.28
Fenvalerate I	2.41	2.37
Fenvalerate II	2.45	2.41
Fenvalerate III	2.53	2.48
Fenvalerate IV	2.58	2.54

[a] Retention time of aldrin = ca 10.37 min.

[b] Retention time of parathion = ca 10.54 min.

[c] Cyano-(4-fluoro-3-phenoxyphenyl)methyl - 3 - (2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (Chemagro Ltd).

[d] Polybrominated biphenyl. Major peak from Firemaster BP6; several other smaller peaks.

dilutions were made to obtain dilute standard solutions and mixtures. Most commonly injected solvents were iso-octane, methanol, or ethyl acetate.

Samples

All substrates analyzed were authentic samples containing weathered residues. Samples were extracted and cleaned up using specific or multiresidue methods commonly employed in this laboratory.

Results

Retention Time and Chromatography

Table 1 is an alphabetic compilation of the pesticides examined in this study; retention times relative to parathion are given. While relative retention times are often difficult to reproduce in other laboratories under temperature-programmed regimes, this list is indicative of elution order, and approximate retention times may be calculated. Table 2 lists the elution order of many of the chlorinated pesticides generally analyzed by ECD; retention times relative to both aldrin and parathion are given. Compounds, by class, responsive to NPD are compiled in Tables 3–7. Typical chromatograms for some organochlorine insecticides, organophosphorus insecticides, carbamate pesticides, organonitrogen herbicides, and some fungicides are shown in Figures 1–5, respectively.

In general, all the examined pesticides chromatographed well and exhibited typical capillary column peak shape; acephate and methamidophos were notable exceptions and showed broad, asymmetrical peak shape. Most compounds had peak widths of less than 0.1 min. The organochlorine and organophosphorus insecticides tended to have wider peaks and also showed some peak tailing (Figures 1 and 2) compared with many of the other classes of pesticides. However, even under severe overloading conditions, most peaks were sharp and minimal tailing was observed. Many compounds that do not chromatograph well by packed column GLC were easily determined with the capillary column. Azinphos-methyl is one example; on packed columns, considerable priming is required to obtain reproducible peak heights, whereas on the capillary column it was consistently reproduced from the first injection.

Capillary columns are recognized for their value in determining thermally labile and easily adsorbed polar compounds. The *N*-methylcarbamates appeared to be quantitatively recovered

Table 3. Retention time relative to parathion of some organophosphorus pesticides and metabolites

Pesticide	RRT[a]	Pesticide	RRT[a]
TEPP	0.15, **0.19**[b]	Malathion	0.98
Dichlorvos	0.27	Chlorpyrifos oxon	0.98
Naled	0.27	Phorate sulfone	0.99
Trichlorfon	0.27	Fenthion	0.99
Methamidophos	0.28	Parathion	1.00
Mevinphos	0.39	Chlorpyrifos	1.00
Acephate	0.43	Dicapthon	1.01
Thionazin	0.53	Pirimiphos-ethyl desethyl	1.02
Dimethoate oxon	0.54	Isofenphos oxon	1.03
Demeton-S	0.54	Crufomate	1.03
Phorate oxon	0.54	Thionazin	1.03
Sulfotep	0.61	Pirimiphos-ethyl oxon	1.04
Phorate	0.62	Trichlornat	1.05
Fonophos oxon	0.62	Bromophos	1.05
Demeton-O	0.66	Pirimiphos-ethyl	1.09
Dimethoate	0.66	Chlorfenvinphos	1.12
Dioxathion	0.71	Isofenphos	1.13
Terbufos	0.72	Methidathion	1.14
Fonofos	0.72	Crotoxyphos	1.16
Diazinon oxon	0.73	Tetrachlorvinphos	1.20
Diazinon	0.76	Fenamiphos	1.25
Disulfoton	0.76	Fensulfothion oxon	1.29
Phorate oxon sulfoxide	0.82	Ethion oxon	1.31
Fenchlorphos oxon	0.82	Fenthion sulfoxide	1.37
Dichlofenthion	0.85	Fensulfothion	1.38
Phosphamidon	0.75, **0.85**	Fenthion sulfone	1.39
Parathion-methyl	0.85	Fensulfothion sulfone	1.42
Chlorpyrifos-methyl	0.86	Ethion	1.43
Malathion oxon	0.87	Carbophenothion	1.49
Oxydemeton-methyl sulfone	0.88	Phosmet	1.64
Parathion oxon	0.89	Azinphos-methyl	1.76
Fenthion oxon	0.89	Phosalone	1.80
Fenchlorphos	0.91	Leptophos	1.81
Fenitrothion	0.93	TOCP	1.88
Phorate sulfoxide	0.94	Coumaphos	2.04
Pirimiphos-methyl	0.96	Bensulide	2.06
Zytron[R]	0.97	Temephos	NR[c]

[a] Retention time of parathion = ca 10.55 min.
[b] Isomers; major peak is boldface.
[c] No response in 40 min with isothermal hold at 250°C.

Table 4. Retention time relative to parathion of some carbamate insecticides and herbicides

Insecticide	RRT[a]	Herbicide	RRT[a]
Methomyl oxime	0.16	EPTC	0.36
Aminocarb phenol	0.35	Butylate	0.41
Oxamyl oxime	0.47	Vernolate	0.42
Propoxur	0.53	Pebulate	0.43
Bendiocarb	0.58	Molinate	0.47
Carbofuran	0.65	Cycloate	0.57
Aminocarb	0.70	Diallate	0.63, 0.65
Bux (technical)	0.61, 0.69, **0.71**, 0.76	Triallate	0.79
3-Ketocarbofuran	0.76		
Pirimicarb	0.81		
Desmethylpirimicarb	0.82		
3-Hydroxycarbofuran	0.84		
Carbaryl	0.85		
Methiocarb	0.93		

[a] Retention time of parathion = ca 10.46 min.

Table 5. Retention time relative to parathion and atrazine of some triazine and chloracetanilide herbicides

Compound	Retention time relative to Parathion[a]	Retention time relative to Atrazine[b]
Propachlor	0.53	0.79
Desethylsimazine	0.56	0.83
Desethylatrazine	0.58	0.85
Atraton	0.66	0.97
Simazine	0.66	0.97
Atrazine	0.68	1.00
Prometon	0.68	1.00
Propazine	0.69	1.02
Metribuzin	0.82	1.21
Cyprazine	0.83	1.22
Ametryn	0.89	1.31
Alachlor	0.89	1.31
Prometryn	0.90	1.34
Terbutryn	0.94	1.38
Cyanazine	0.97	1.43
Metolachlor	0.99	1.47
Hexazinone	1.52	2.24

[a] Retention time of parathion = 10.45 min.
[b] Retention time of atrazine = 7.09 min.

from the capillary column, except when the insert became contaminated (see *Discussion*). Since a flame ionization detector was not used, the degree of breakdown to their phenols could not be determined with NPD. Aminocarb, which contains a nitrogen atom in its phenol, showed only minimal response to its phenol and this may have been due to contamination of the standard solution. Based on the response of the carbamate insecticides, it appears that minimal thermal breakdown was occurring. Similarly, fresh standard solutions of *N*-phenylurea herbicides indicated only one peak, whereas older standards demonstrated considerable breakdown. Phenolic and acidic compounds, such as dinoseb, 4-hydroxychlorothalonil, dicamba, and metalaxyl acid, could be chromatographed without resorting to methylation. Their responses were lower than anticipated, particularly with a well used column, and after methylation better chromatography and response was obtained. Their presence in samples could be determined

Table 6. Retention time relative to parathion of phenylurea and other herbicides

Compound	RRT[a]	Compound	RRT[a]
Phenylurea herbicides:			
3,4-Dichloroaniline	0.39	Diquat (reduced)	0.52
Tebuthiuron	0.43	CIPC	0.59
Monuron	0.48	Paraquat (reduced)	0.60
Diuron	0.68	Sulfallate	0.62
Metobromuron	0.80	Trifluralin	0.64
Linuron	0.95	Terbacil	0.74
Chlorbromuron	1.09	Dinitramine	0.78
Chloroxuron	1.50	Dinoseb-OMe	0.82
Miscellaneous herbicides:		Bromacil	0.93
AMPA[b] N-TFA-$(OMe)_2$	0.24	2,2-Diphenylacetamide	0.99
Allidochlor	0.30	Desmethyldiphenamid	1.00
Dichlobenil	0.33	Diphenamid	1.03
Glyphosate N-TFA-$(OMe)_3$	0.44	Nitrofen	1.33

[a] Retention time of parathion = ca 10.45 min.
[b] Aminomethyl phosphonic acid, glyphosate metabolite.

Table 7. Retention time relative to parathion of some fungicides

Compound[a]	RRT[b]	Compound[a]	RRT[b]
Tecnazene	0.53	Furalaxyl	1.14
Dichloran	0.65	Triadimenol	1.15
Quintozene	0.71	Carboxin	1,28
Chlorothalonil	0.73	RE-26745	1.37
4-Hydroxychlorothalonil-OMe	0.77	Binapacryl	1.38
Vinclozolin	0.87	RE-20615	1.43
Metalaxyl	0.90	Galben	1.50
Triadimefon	1.02	Difolatan	1.54
Meclozolyn	1.04	RE-26940	1.58
Metalaxyl acid	1.06	Metalaxyl acid-O-PFB	1.61
Captan	1.07	Iprodione	1.68
Quinomethionate	1.13		

[a] See Table 1 for compound identity.
[b] Retention time of parathion = 10.45 min.

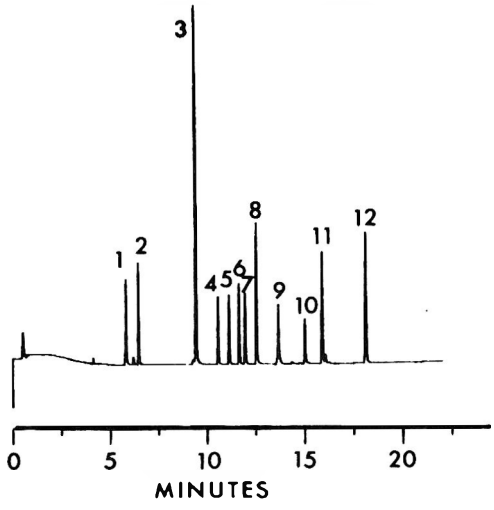

Figure 1. ECD chromatogram of 12 organochlorine insecticides. See text for operating conditions. Peak identity, ng: (1) α-BHC, 0.1; (2) lindane, 0.1; (3) aldrin, 0.1; (4) oxychlordane, 0.1; (5) γ-chlordane, 0.1; (6) α-chlordane, 0.1; (7) *trans*-nonachlor, 0.1; (8) *p,p'*-DDE, 0.2; (9) *p,p'*-DDD, 0.2; (10) *p,p'*-DDT, 0.2; (11) photomirex, 0.2; (12) mirex, 0.2.

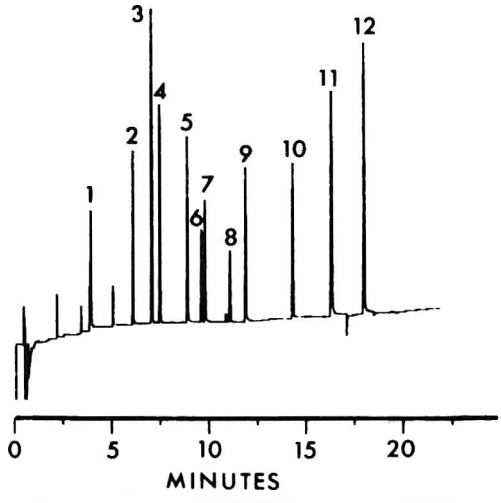

Figure 2. NPD chromatogram of 12 organophosphorus insecticides. Peak identity, ng: (1) mevinphos, 1; (2) phorate, 1; (3) fonofos, 1; (4) diazinon, 1; (5) fenchlorphos, 2; (6) malathion, 1; (7) parathion, 1; (8) chlorfenvinphos, 2; (8) tetrachlorvinphos, 5; (10) ethion, 1; (11) phosmet, 5; (12) phosalone, 5.

without derivatization but results were better after chemical modification. Furthermore, it was observed that some compounds, such as the phthalimide fungicides, respond better to one detector and their absence on another detector is not indicative of poor chromatography.

Reproducibility

There is conflicting evidence in the literature (4) concerning the reproducibility of CGC systems. The reproducibility of retention time, peak area, and peak height for both continuous injections of standards and for standards interspersed with other standards or samples during overnight or longer runs was examined. The latter was taken as a better indicator of actual use although these values had slightly larger errors. Table 8 shows the reproducibility of retention time for representative compounds in each class. Over several days of continuous use, retention times of standards generally varied by ±0.01 min while those in actual samples varied by ±0.02 min. Only a few compounds, such as acephate, showed asymmetric peak shape and in these cases their retention times over a concentration series varied by ±0.05–0.1 min. Retention time was confirmed by injecting standards at concentrations corresponding to residue levels found in the samples. With increasing use and

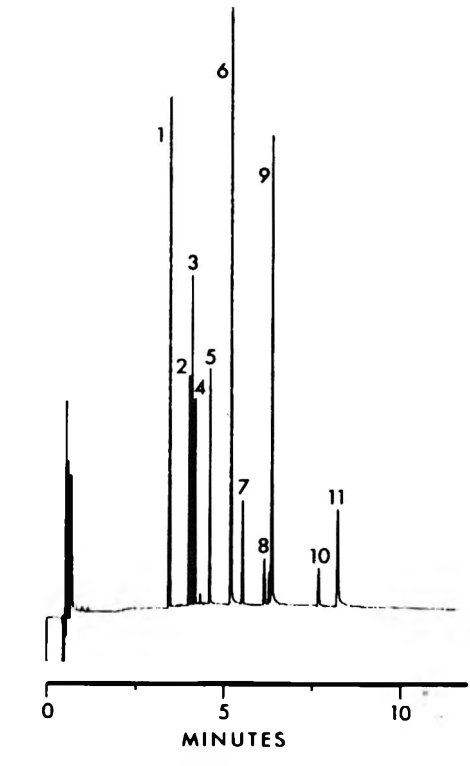

Figure 3. NPD chromatogram of 11 carbamate pesticides. Peak identity, ng: (1) EPTC, 0.5; (2) butylate, 0.5; (3) vernolate, 1; (4) pebulate, 1; (5) molinate, 1; (6) propoxur, 5; (7) cycloate, 1; (8) diallate, 1; (9) carbofuran, 5; (10) triallate, 1; (11) carbaryl, 5.

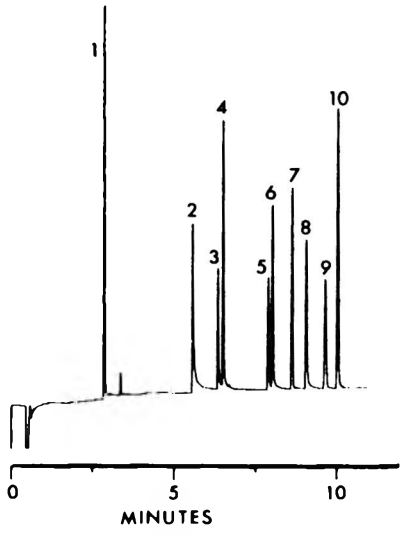

Figure 4. NPD chromatogram of 10 organonitrogen herbicides. Peak identity, ng: (1) allidochlor, 2.5; (2) de-ethyl atrazine, 1; (3) simazine, 1; (4) atrazine, 1; (5) metribuzin, 1; (6) cyprazine, 1; (7) alachlor, 5; (8) terbutryn, 1; (9) metolachlor, 5; (10) diphenamid, 5.

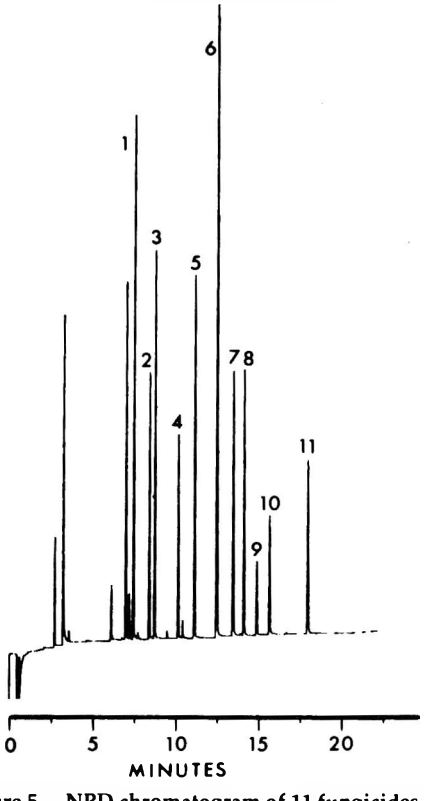

Figure 5. NPD chromatogram of 11 fungicides at 10 ng each. Peak identity: (1) chlorothalonil; (2) vinclozolin; (3) metalaxyl; (4) meclozolyn; (5) furalaxyl; (6) carboxin; (7) RE-26745; (8) RE-20615; (9) Galben; (10) RE-26940; (11) iprodione.

Table 8. Reproducibility of retention time for representative pesticides, using the automatic liquid sampler ($n > 6$)

Compound	Mean retention time, min	SD	CV, %
Butylate	4.247	0.002	0.039
Atrazine	6.804	0.001	0.008
Lindane[a]	7.864	0.010	0.13
Carbaryl	8.804	0.001	0.010
Metalaxyl	9.313	0.001	0.008
Alachlor	10.319	0.001	0.008
Parathion	10.361	0.001	0.012
Ethion	14.952	0.002	0.012
p,p'-DDT[a]	17.123	0.012	0.071
Phosalone	18.689	0.001	0.008
Mirex[a]	20.111	0.011	0.056

[a] $n = 18$, injected over one week, interspersed with samples.

age of the column, retention times tended to be longer but the relative retention times remained fairly constant. Wear on the septum was not indicated as a major source of error in retention time reproducibility over several hundred injections.

Table 9 shows the comparative reproducibility of peak height and area for a series of noncontiguous standard injections as determined by the Level 4 terminal. Overall, peak height was generally more reproducible although both methods of quantitation indicated comparable results. For short term (one day) operation, integration results were very reproducible with a coefficient of variation of less than 10%; however, over several days the detector and chromatographic performance change (see *Discussion*) and this is reflected in the data. Periodic monitoring with standard injections every 10–30 samples and recalibration of the algorithm is recommended.

Detector Performance

The electron capture detectors on the Hewlett-Packard 5700, 5830, and 5880 series gas chromatographs have a similar design. Experience with these instruments in this laboratory and others (2) has shown that the 5830 ECD was nonlinear, whereas the 5700 ECD was linear both within and between attenuations. In this study, the 5880 ECD was linear over the 0.001–1.0 μg/mL concentration range examined. Regression analysis indicated that both peak height and area were linear and R^2 values >99% were found. Peak height generally produced better correlations of linearity which may have been due to column overloading effects and the slight tailing of the organochlorine compounds on the

Table 9. Comparative reproducibility of peak height and peak area for representative pesticides, using automatic liquid sampler and Level 4 terminal algorithm ($n > 6$)

Compound	Concn, μg/mL	Peak ht		Peak area	
		Mean	CV, %[a]	Mean	CV, %[a]
Butylate[b]	10	1834	3.5	2639	3.6
Atrazine	1.0	43	4.7	119	4.5
Lindane[c]	0.01	179	29	ND[d]	
Carbaryl	10	27	21	105	18
Metalaxyl	10	24	18	63	16
Alachlor	1.0	5.9	5.5	18	4.6
Parathion	1.0	50	5.6	212	10
Ethion	1.0	103	5.7	497	4.7
p,p'-DDT[c]	0.02	65	41	ND	
Phosalone	5.0	163	7.3	1038	5.1
Mirex[c]	0.02	365	6.5	ND	

[a] Coefficient of variation.
[b] All compounds determined on NPD, except organochlorines were analyzed by ECD.
[c] Determined by ECD. Mean of 13 replications conducted over one week with standards interspersed with samples.
[d] Not determined.

SE-30 capillary column. Some variations in response were observed at individual concentrations but these were probably due to dilution errors or chromatographic performance.

As in most past ECD performance trials, the 5880 ECD was durable and consistent for long periods. The CGC system was located in close proximity to chlorinated solvents, and although the baseline offset increased considerably over the day, it was not noticeable on the chromatograms and no detrimental effects have been observed. Over a period of several days the ECD response to organochlorine compounds changed to produce coefficients of variation of 10–50% (Table 9) with respect to peak height. This variation was determined to have occurred because of the injection of over 100 fish samples with deposition of lipid material on the capillary insert and was not due to the stability of the ECD.

The nitrogen-phosphorus thermionic detector is generally recognized to be less stable and less reproducible than electron capture or flame photometric detectors, yet it has a very important place in pesticide residue analysis. Our experience has shown there are wide differences in the stability, performance, and life-time (up to 6 months) of the NPD collector bead. Some collectors work well for over 2 months with minimal adjustment while others require constant maintenance. The NPD is left in operation (voltage and gases on) at all times and the voltage offset is adjusted only when required. Occasionally, peak heights increase with injections (conditioning), or decrease in sensitivity with use (contamination), while other collectors produce fairly constant response. During overnight injections, the overall coefficient of variation of peak height is similar to that found with ECD but sensitivity trends are quite obvious. Performance is generally better with phosphorus compounds than with nitrogen-containing pesticides. Often the best results are obtained with a well used and conditioned collector, and under these conditions quantitative reproducibility was <30%. Chlorinated solvents tend to reversibly sensitize the collector bead. Much more attention must be given to performance evaluation of this detector and frequent standard injection and recalibration is required.

The NPD was linear to all the tested organophosphorus and organonitrogen pesticides over the range 0.1–10 μg/mL. Linearity results were similar to those found with the ECD, and again peak height was determined to be marginally better for quantitation. Many of the organophosphorus pesticides exhibited peak tailing which could affect the algorithm calculating areas.

Depending on the molecular and chemical structure of the pesticide, the detector response with the NPD can be used to predict approximate response factors as discussed by Holland and Greenhalgh (18). Unfortunately, this is not true in all cases, and lower responses with some compounds may have been due either to the detector response to the chemical or to poor chromatographic performance. The phthalimide fungicides (captan, folpet, captafol) show very poor NPD response yet they have excellent ECD sensitivity under identical conditions; the dicarboximide fungicides (iprodione, meclozo-

Table 10. Comparison of quantitation data on diphenamid and desmethyl diphenamid in tobacco extracts, using packed column with Hall detector and capillary system with NPD

	Concn in sample, μg/g			
	Diphenamid		Desmethyl diphenamid	
Sample No.	Cap. NPD	Packed Hall	Cap. NPD	Packed Hall
1	28	29	8.5	9.4
2	8.3	8.5	5.1	6.5
3	5.1	4.0	3.9	3.7
4	2.9	2.4	2.6	2.6
5	0.88	0.61	0.43	0.37

lyn, vinclozolin) show the expected NPD response.

Notwithstanding these problems, excellent correlations were obtained in quantitative comparisons of capillary and packed column GLC results. Table 10 shows a comparison of analysis figures for diphenamid and desmethyl diphenamid in tobacco samples. Tobacco was extracted and cleaned up as described by Sirons et al. (19); final extracts were examined by packed column GLC with a Hall detector and with the capillary system and N-P detection.

Discussion

Many pesticide residue laboratories are required to analyze a large number of pesticides and their metabolites in a variety of substrates of animal, vegetable, or mineral origin. Typically, samples originate from programs for monitoring and surveillance, quality assurance of food, agricultural or soil commodities, registration or recommendations for pesticide use, or misapplication or health problems. Concentrations range from ppb levels in water, to ppb–ppm levels in food samples, and % composition in spray solutions or formulations.

Samples may be analyzed for a single pesticide, or a general screening may be required. Two analytical approaches are thus possible: Specific methods may be applied to one or more analytes in a single substrate, or a general multiresidue methodology may be applied. Because of the variety of chemical and physical properties of pesticides, these procedures are often divided into convenient, and where possible, multiresidue classes. Hence, optimized recoveries are obtained using different methodologies for extraction and cleanup.

Determinations are usually conducted by gas-liquid chromatography using a variety of columns and detectors such as electron capture, flame photometric (phosphorus and sulfur), nitrogen-phosphorus, and Hall and Coulson (oxidative and reductive modes). Specialized GLC columns and derivatization techniques are often required to enhance chromatographic performance as well as detector sensitivity and specificity.

Despite these analytical regimes, several problems persist. A large number of different GLC columns and conditions are required for analysis and separation of certain classes of pesticides, and chemical modification is often required for thermally labile or nonsensitive analytes. Analysis is usually geared to the small number of compounds in a specific class and often other residues may go unnoticed or cause an interference in the determination. Moreover, the increased demands for greater capability in number of samples and analytes determined necessitates some automation and computerization.

Hence, a single capillary gas chromatographic column under a general oven-programmed scheme was evaluated for the qualitative and quantitative analysis of pesticide residues. Since most pesticides contain at least one chlorine, phosphorus, or nitrogen atom, determination was evaluated with electron capture and nitrogen-phosphorus detectors.

Most of the compounds examined had unique retention times (RT) under the described conditions, and the reproducibility of RT values (Table 8) was sufficient that the 15 m SE-30 capillary column could be used for qualitative analysis. However, because of asymmetric peaks, concentration differences, and variable responses from sample matrices, such determinations are only semiqualitative. Depending on the analytical workup, it was found that CGC provided a rapid screening method to determine possible residues, as evidenced by response and retention time, that should be confirmed under other analytical conditions or with other GLC columns or detectors. On the other hand, preliminary screening by GLC and confirmation of peak identity by its characteristic CGC retention time can also be beneficial. Hence, the 2 techniques are complementary and each has a role in the determination step. Interpretation and experience with the chromatographic system, substrates, and residues are still important in final analysis.

For example, a typical extraction of a water or soil sample with methylene chloride will recover, although not necessarily quantitatively,

a large number of pesticides of different classes. Analysis of these extracts by CGC with NPD will indicate numerous pesticide residues that under other analytical regimes could require 3 or more specific determinations. Experience is essential in the interpretation of this CGC data and subsequent confirmation is often essential. For example, atrazine is a somewhat ubiquitous residue in mineral environmental samples of many agricultural areas; its presence could interfere with detecting several other pesticides.

The use of CGC in studies with compounds of known use or contamination is also very beneficial. The high resolution of the capillary column allows separation of many of the co-extractives from the analyte and clear windows are much easier to obtain. Consequently, less rigorous cleanup of samples may be required but consideration must be given to overloading the column or contaminating the CGC system. With packed GLC columns, early-eluting peaks near the solvent front may tail badly and interfere with the determination of volatile pesticides. With the capillary system, these peaks are not as pronounced and are often absent, and as such, better quantitation of early peaks (e.g., HCB, lindane) is possible.

Multiresidue methods for the extraction and cleanup of many chlorinated pesticides are available, and most extracts currently analyzed by packed GLC/ECD pose no background problems with the CGC/ECD system. Indeed, the superior resolution of CGC permits qualitation and quantitation at levels which are 10 times lower compared with packed column determination. Electronic integration and data manipulation suggest that residues are present but these data must still be interpreted and confirmed. Unfortunately, fewer multiresidue methods and specific cleanup methodologies exist for the organonitrogen compounds. In many of these analyses, specific methods are applied for the determination of known or suspected pesticides; other residues may inadvertently be recovered as well. Control samples are much more essential to ensure qualitative analysis. Extraneous peaks can also be qualitatively examined based on relative retention time for the presence of other residues. The recovery of other pesticides with these specific methods is often unknown and it should be checked and optimized if possible.

Crude and cleaned up extracts from authentic samples were examined with CGC/NPD. Retention time windows were found, sometimes after appropriate cleanup, for known analytes. Numerous co-extractives were found in all sample matrices, and hence untreated samples are essential if a screening procedure is used. The NPD did provide a high degree of selectivity for many matrices. Crude onion extracts masked a large portion of the chromatogram from packed and capillary columns with ECD but with the NPD only a few minor peaks were observed; similarly, crude extracts of conifer foliage were remarkably clean with the NPD. Tobacco samples required coagulation or column cleanup to reduce the background; crude potato extracts showed many extraneous peaks. Water and other matrices showed a few extraneous peaks corresponding to those commonly encountered with packed column FPD (phosphorus detection). No detrimental column effects were observed as the result of crude extract injections but, as discussed below, syringe carryover and contamination of the capillary insert created problems. Continuous injection of samples did not change the detector response any more than did standard injections. Conditioning of the bead and CGC system with sample extracts often was beneficial in obtaining good chromatographic performance. When contamination became a problem, the insert was cleaned and responses returned to initial levels. Continuous monitoring of the system with the analyte is imperative for quantitative results.

One of the most critical factors in optimizing the CGC performance is the condition of the capillary insert. Repeated injections of samples, both with and without cleanup, caused a noticeable buildup of co-extractive material on the insert. For example, the large coefficient of variation in peak height for *p,p'*-DDT (Table 9) was demonstrated to arise from the deposition of lipid material on the insert. Over several weeks, the ECD response to *p,p'*-DDT decreased to 25% of its original value. Thus, adsorptive and degradative processes can occur at the most critical area for capillary column performance, the introduction of the analyte to the column. Similarly, injection of unclean crop extracts resulted in contamination of the insert with the result that the thermally labile *N*-methylcarbamates showed no response by the NPD. Ultrasonic cleaning with Decon or Contrad 70, or similar nonphosphorus-containing detergents, and if necessary physical cleaning of the insert, was sufficient to restore the chromatographic performance. Hence, continuous monitoring of the system is necessary to ensure optimum performance. Short term variations may be overcome with the recalibration process.

After several months of continuous use, the column showed some deterioration from either removal of liquid phase or contamination by co-extractives near the injector. Often, breaking off the first 15–20 cm of the capillary column restored the chromatographic performance. Retention times are only slightly reduced but the relative retention times remain quite similar (±0.02 relative to reference).

Problems were encountered with syringe carry-over when the automatic liquid sampler (ALS) is used. Typically, the ALS rinses the syringe 5 times with the next sample before injection. Frequently, similar patterns of background or traces of analytes were observed in the next injection. For example, following injection of a 10–50 μg/mL organophosphorus standard mixture, traces to 2% of the response were observed in a subsequent isooctane blank injection. With some compounds, as much as 10% carry-over was observed. The cause was determined to be insufficient rinsing of the syringe and not adsorption/desorption from the insert or ghosting from the column. Choice of solvent for rinsing may be important, as is the number of rinses. This problem must be recognized when routine samples of unknown, and varying concentrations, are analyzed.

Conclusion

Capillary gas chromatography offers many advantages to the pesticide residue analyst. The high resolution of the column allows separation and more positive identification as based on retention times. The reproducibility of retention time permits qualitative identification of pesticides; however, CGC complements packed columns with specific detectors and each should be used for analysis and confirmation. The column resolution facilitates separation of known analytes from co-extractives, and as such, improved confirmation of identity and better detection limits are obtained. For many compounds, quantitation in samples may be accomplished at a decade or lower compared with packed columns.

The high resolution may present some problems if the somewhat nonspecific detectors (ECD, NPD) are used. With some sample extracts, numerous extraneous peaks are evidenced and for general screening purposes may be incorrectly identified if cleanup steps are not used or if peaks are not confirmed with alternative detectors or via chemical modification. With some matrices, 20 or more pesticides may be identified as trace residues based solely on retention time. Hence, untreated sample extracts are important as reference materials. Validated residue methodology is also important in achieving accurate data; extraction, partitioning, and cleanup steps increase the confidence that the residue is truly present. Unfortunately, such methods do not exist, particularly for multiresidue applications, for many of the newer pesticides.

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Determination of Acrylonitrile in Foods by Headspace Gas-Liquid Chromatography with Nitrogen-Phosphorus Detection

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A general procedure is described for the determination of acrylonitrile (AN) in foods such as margarine, honey butter, cold-pack cheese, and peanut butter, which are likely to be packaged in AN-based plastic. The entire sample is blended with water and salt at <5°C, aliquots are sealed in crimp-top vials, and the vials are equilibrated in a boiling water bath. The headspace is sampled by using a heated syringe, and AN is determined by gas chromatography with a nitrogen-phosphorus selective detector. The inclusion of propionitrile as an internal standard allows quantitation of AN with detection at 4, 4, and 10 ppb for margarine, honey butter, and cold-pack cheese, respectively. A peak corresponding to about 5 ppb apparent AN in all non-AN-packaged peanut butter samples examined limits detection in peanut butter to about 15 ppb. The coefficients of variation at 20 ppb for margarine, honey butter, cold-pack cheese, and peanut butter were 7.5, 8.3, 7.3, and 10.2%, respectively.

The carcinogenic activity of acrylonitrile (AN) has recently been demonstrated in a comprehensive study (1). This report, combined with previous reports, has resulted in several analytical approaches to determine trace quantities of AN in AN-based polymers as well as the usual food simulants. These techniques, based mainly on gas chromatographic procedures, have become increasingly specific and sensitive over the last few years.

Since 1976, residual AN has been determined by the gas chromatographic-headspace technique in a number of different matrices; in polymers by using a flame ionization detector (FID) (2); in food simulants by using a nitrogen-specific thermionic detector (3); and in plastic resins (4, 5), olive oil (4), and beverages (5) by using a thermionic nitrogen-phosphorus (N-P) selective detector.

AN has also been determined in diluted paint, in acetone extracts of charcoal, and in water (6) by direct injection, using a N-P selective detector with an electrically heated rubidium silicate-based glass bead. Gas chromatography-quadrapole mass spectrometry (GC-MS) was used to confirm AN levels. Similarly, Brown et al. (7) used the N-P detector and direct injection to determine AN in heptane, 3% acetic acid, 8 and 50% ethanol, and water as food simulants. The levels in the simulants were subsequently confirmed by GC-quadrupole MS by McNeal et al. (8) following azeotropic concentration. Stampa and Imhof (9) used the headspace technique, propionitrile internal standard, and an N-P detector to determine AN as low as 10 ppb in studying the migration of AN from plastic resins into simulants.

It is apparent that headspace determination with N-P detection is the preferred analytical approach to determine AN in food simulants, homogeneous liquid samples, and plastic resins. No determinations or methods have been described, however, for determining AN as a migrant from AN-based plastic into foods such as margarine, peanut butter, cheese products, or other spreads that are likely to be packaged in AN-based plastic containers. In these semisolid foods, migrant AN would be expected to be distributed inhomogeneously within the contents of the container, with the concentration of AN highest next to the container walls. Thus, to estimate the AN concentration of the total contents, the entire sample or a representative fraction must be made homogeneous before the analytical sample is taken.

This report describes a procedure for determining AN in semisolid foods such as margarine, honey butter, cold-pack cheese, and peanut butter. In developing methodology, one of our goals was that the procedure be applicable to a wide range of foods. Thus, the general procedure could be used for foods other than those studied. In the proposed procedure, the entire contents of the container are blended to give a homogeneous aqueous saline slurry, an aliquot is sampled, and AN is determined by headspace gas chromatography with N-P detection, using propionitrile (PN) as internal standard. Samples of 5 types of food packaged in AN-based plastic containers and the containers themselves were analyzed for AN. Some results were confirmed by high resolution GC-MS.

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METHOD

Apparatus

(a) *Gas chromatograph.*—Varian Aerograph Model 2100, or equivalent, equipped with 6 ft × 4 mm id U-shape glass column packed with 80–100 mesh Chromosorb 102 (Johns-Manville Co. Ltd) and fitted with a Tracor Model 702 nitrogen–phosphorus (N-P) selective detector, or equivalent, adapted for on-column injection, and with column end fitted into detector base. Operating conditions: temperatures (°C)—injector 200, column 140, detector 225; helium carrier gas at 40 mL/min; hydrogen at 3.0 mL/min; air at 110 mL/min; stabilized source heating current adjusted to give ca 20 pA (50% full scale deflection at 4×10^{-11} amp/mV); polarizing voltage, high setting. Desensitize N-P detector for 2 min after injection. Retention times for acrylonitrile (AN) and propionitrile (PN) should be 6–7 and 9–10 min, respectively. An injection of 3.22 ng AN (4.0 μL of 0.806 μg/mL) should give >50% full scale deflection at 64×10^{-12} amp/mV.

(b) *Syringe.*—1 mL gas-tight syringe Model 1001 (Hamilton Co.) equipped with syringe heating mantle Model 87204 (Hamilton Co.). Heat syringe with steam. Use boiling water bath to supply steam and water aspirator to draw steam through syringe, or use steam generator. Connect syringe with ball joints and clips or tubing connectors. Check heating efficiency in mantle by substituting thermometer and cork for syringe. Temperature should be ≥98°C. Use 2 in. 22 gage needle.

(c) *Water bath.*—Boiling water bath, Boekel Model 1487, or equivalent, modified to hold at least 16 headspace vials as follows: Replace concentric rings with circular metal plate with ¾ in. wide, 1 in. deep notches on perimeter to receive necks of sample vials. Fit protruding bolt or screw in center of disk. A 5 in. diameter plate can hold 4 vials. Hold vials in place with elastic band over bolt. Do not use same water bath used for heating syringe mantle.

(d) *Headspace sample vials.*—30 mL Hypovials (Pierce Chemical Co.), or equivalent, sealed with Teflon-laminated silicon rubber disks (Tufbond, Pierce Chemical Co.), or equivalent. Discard disk after single use. Crimp headspace sample vials tightly so aluminum cap cannot turn.

(e) *Pipet.*—10 mL, Mohr or serological, for transferring peanut butter or margarine slurry, with tip completely cut off; for transferring honey butter or cheese slurry, cut off tip so internal diameter is 1–2 mm.

Reagents and Materials

Caution: Acrylonitrile is a carcinogen. Observe necessary safety precautions. Carry out all manipulations and dilutions in fume hood.

(a) *Margarine, honey butter, cold-pack cheese, peanut butter.*—As purchased in local retail stores. Packaged in glass (or non-AN-based plastic) as available.

(b) *p-Methoxyphenol.*—Monomethyl ether of hydroquinone (MEHQ). Available from Aldrich.

(c) *Acrylonitrile (AN).*—Available from Aldrich.

(d) *Propionitrile (PN).*—Available from Aldrich.

(e) *Distilled water.*—Add MEHQ; if necessary, stir with Teflon-covered stirring bar to dissolve to give 1% (w/v) solution.

(f) *Absolute ethanol.*—Add MEHQ and dissolve to give 1% (w/v) solution.

(g) *Sodium chloride.*—Analytical reagent.

(h) *Standard solutions.*—Refrigerate.

(*1*) *AN stock solution.*—0.806 μg/mL. Pipet 1 mL (0.806 g) AN into 100 mL volumetric flask containing about 50 mL ethanol, dilute to volume with ethanol, and mix. Pipet 1 mL aliquot into 100 mL volumetric flask, dilute to volume with water, and mix. Pipet 1 mL aliquot into 100 mL volumetric flask, dilute to volume with water, and mix.

(*2*) *PN stock solution.*—0.772 μg/mL. Prepare as (*1*) above.

(*3*) *Preparation of AN spiking standards.*—From stock solutions (*1*) and (*2*) above, prepare 6 standard solutions each containing 386 ng PN/mL and 0, 40.3, 80.6, 161.42, 241.8, and 403 ng AN/mL by pipetting 5 mL PN solution into each of six 10 mL volumetric flasks and 0, 0.5, 1, 2, 3, and 5 mL AN solution into each flask. Dilute to volume where necessary with water.

(*4*) *PN internal standard solution.*—0.386 μg/mL. Pipet 5 mL PN stock solution (*2*) above into 10 mL volumetric flask. Dilute to volume with water and mix.

Preparation of Calibration Standards

If glass-packaged or non-AN-based plastic-packaged samples of the food to be analyzed are not available, AN can be stripped from AN-packaged food by the following procedure: Weigh 200–300 g (X g) of food into 2 L round-bottom flask, weigh flask and contents (Y g). Add 2X g water (no MEHQ) and evaporate water on rotary evaporator until weight approximating Y g is obtained. Separately add 2 portions (each

= X g) of water, evaporating to approximately Y g after each addition. Add X g of water (with 1% MEHQ) and mix. Decant into tared blender and determine weight. Add 1/4 this weight of NaCl and blend 1 min.

For glass-packaged or non-AN-based plastic-packaged food, weigh 200–300 g (X g) food into blender, add X g distilled water (with 1% MEHQ) and X/2 g NaCl. Blend 5 min.

Using suitable Mohr pipet and water aspirator, fill Mohr pipet almost to top with food–water–salt slurry, from blender. Wipe outside of pipet tip with disposable tissue, and add exactly 10.0 g slurry into each of twelve 30 mL headspace sample vials, blending slurry briefly to mix between each sampling. To each of these 12 vials add 1 mL of the appropriate solution (h)3 to give PN concentrations of 96.5 ppb and AN concentrations of 0, 0, 10.1, 10.1, 20.2, 20.2, 40.3, 40.3, 60.5, 60.5, 100.8, and 100.8 ppb, with respect to the food. Immediately cap vial with closure after adding each 1 mL aliquot. Mix flask by rotation to ensure homogeneous slurry after adding each 1 mL aliquot.

Preparation of Calibration Curves

Equilibrate in boiling water bath for 2 h and sample headspace as described below under *Headspace Technique* after exactly 2 h in bath.

Using chromatogram of blank sample as guide, draw baseline and estimate peak heights of AN (H_a) and PN (H_p) to nearest 0.1 mm on chromatogram, correcting for any interfering peak in blank sample. Determine ratio H_a/H_p for 10 headspace injections. Plot H_a/H_p vs ppb AN and draw best line through points.

Preparation of Sample

Keep all samples to be analyzed, NaCl, and water (with 1% MEHQ) under refrigeration before preparation of food–water–salt slurry. Chill blender container in freezer. After blending sample, keep blender container and contents in ice bath while sampling except when blending between samples.

Transfer as much as possible of the contents of AN-based plastic-packaged food to chilled, tared blender taking care not to abrade plastic container. Determine weight of food sampled (X g). Add X g cold water and X/2 g NaCl and blend 5 min or until homogeneous. Do not overblend as sample will warm up.

Using suitable Mohr pipet and water aspirator, fill pipet almost to top with food–water–salt slurry from blender. Wipe outside of pipet tip with disposable tissue and add exactly 10.0 g slurry in duplicate into two 30 mL headspace sample vials, blending slurry briefly between samples. To each vial add 1 mL PN internal standard solution (4). Immediately cap vial with closure after adding each 1 mL aliquot. Mix flask by rotation to ensure homogeneous slurry after adding internal standard.

Equilibrate in boiling water bath for 2 h and sample headspace as described below under *Headspace Technique* after exactly 2 h in bath.

Headspace Technique

For at least 15 min before sampling, heat syringe mantle and syringe with steam. Keep plunger in steam-heated test tube. About 7 min before each sampling, briefly flush syringe with nitrogen. Ensure that needle is clear of bits of septum by passing wire into needle.

Use following techniques to sample headspace: Replace hot plunger in syringe and depress fully. Disconnect mantle from steam source, freeing heated syringe. Pierce septum of vial with needle about 1 cm. Withdraw plunger slowly (5 s) to about 1 mL. (A mark on the plunger may be helpful.) Do not pump plunger. Wait 10 s to let barrel fill. Withdraw needle and immediately inject into gas chromatograph. Withdraw needle from gas chromatograph and reconnect mantle to steam source and prepare for next sample. Identify AN and PN from retention times.

Injection of margarine, honey butter, and cold-pack cheese headspace may be made every 15 min. For peanut butter only, a large late-eluting peak (retention time about 3.8 relative to PN) will interfere after 2 injections. Therefore, after 2 injections raise the oven temperature to 200°C for 10 min. After cooling and re-equilibration of oven at initial temperature, 2 more samples may be injected.

Calculation

Measure peak heights of AN (H_a) and PN (H_p) on chromatogram and determine average ratio H_a/H_p from duplicate samples. Determine ppb AN from calibration curve.

Results and Discussion

Initial studies in the development of suitable methodology for AN in foods as well as plastics were carried out using a Coulson electrolytic conductivity detector (CECD) operating in the catalytic nitrogen mode. With the acquisition of a N-P thermionic detector with the electrically heated Rb silicate glass bead, much better sensitivity and peak symmetry was observed. Gen-

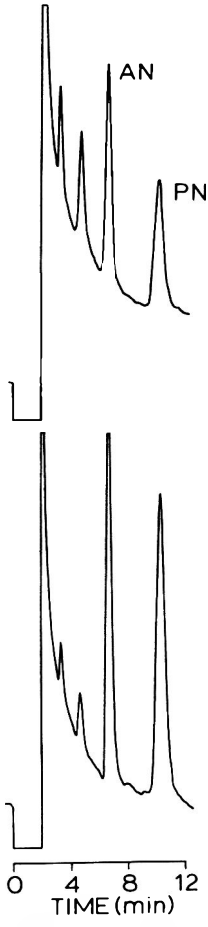

Figure 1. Sensitivity comparison at 8×10^{-12} amp/mV N-P detection): top, 4 μL direct injection of 80 ppb AN and 78 ppb PN; bottom, 1 mL headspace at 100°C from 10 mL water with 2 g salt, 16 ppb AN, and 39 ppb PN.

erally, at maximum practical sensitivity, the N-P detector was about 20 times more sensitive than the CECD. In addition to the Rb silicate beads supplied with the instrument, similar beads were prepared according to a published procedure (10). Little difference in sensitivity or stability was noted with these beads. The greater sensitivity of the headspace procedure compared with direct injection for the determination of AN in aqueous solution is shown in Figure 1. In the headspace determination, 1 mL of the headspace over 10 mL of an equilibrated (100°C) 16 ppb aqueous solution with 2 g salt gives a response almost double that of a 4 μL injection of an 80 ppb solution. Thus for equal responses of normal headspace (1 mL) and direct (4 μL) injections, the headspace procedure is nearly 10 times more sensitive.

Because our analytical interest concerned foods likely to be packaged in AN-based resins, such as margarine, cold-pack cheese, peanut butter, and honey butter and other spreads, it was necessary to develop a method to accommodate the expected uneven distribution of AN in the sample. Preliminary investigations on the viscous or semisolid foods mentioned confirmed higher concentrations of AN in wall samples compared with those taken from the middle. Therefore, any valid method for migrant AN must include a step to homogenize an inherently inhomogeneous sample so that the analytical aliquot will accurately reflect the average AN level of the food package contents. To obtain the required representative analytical aliquot for headspace equilibration, the entire sample was homogenized rather than a representative fraction. This avoided the problems involved in taking a representative pie-shape segment from a jar or circular tub or removing a half or quarter section from a rectangular container. This approach is almost impossible with highly viscous samples such as honey butter. Blending the entire contents (X g) of the chilled container with an equal weight of ice-cold water containing 1% MEHQ and 0.5X g sodium chloride in an ice-cold blender gave a slurry that could be easily sampled and cleanly transferred to the tared headspace vial by using a Mohr pipet. To accommodate different viscosities, the pipet tips were cut to give different diameters. The use of a chilled blender, sample, water, and salt sought to minimize evaporative losses. Because MEHQ was used to stabilize the neat AN, it was felt prudent to include MEHQ in the standard AN solutions. Similarly, MEHQ was incorporated in the aqueous food slurries that were heated during equilibration.

The addition of electrolytes to lower the solubility of the dissolved analyte and increase the partition between headspace and aqueous media has been described by several workers (11, 12). In our samples, it also enhanced the partition to give greater sensitivity. For example, for 10 mL water spiked at 40 ppb AN and 100 ppb PN, the headspace gas chromatographic response increased by factors of 2.6 and 2.9, respectively.

In addition to "salting-out," an increase in temperature will also increase the concentration of volatile analytes in the headspace (12). For our analysis it was convenient to use a boiling water bath with the headspace vials suspended in a slotted lid so that only the aluminum cap and septum were not directly heated. To prevent condensation of the headspace volatiles onto the

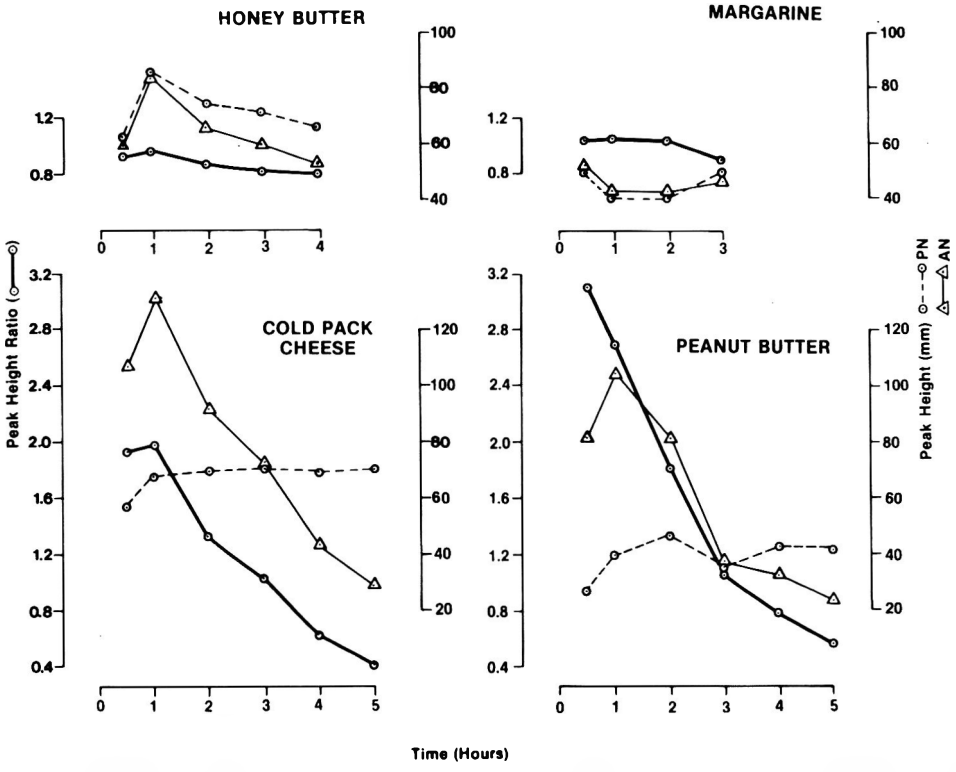

Figure 2. Equilibrium-reaction curves for 4 foods: honey butter and margarine spiked at 50 ppb AN and 100 ppb PN, cold-pack cheese at 100 ppb AN and PN, and peanut butter at 100 ppb AN and 50 ppb PN.

cold syringe barrel or other syringe parts, it was necessary to heat the syringe to the water bath temperature. If condensation occurs, small quantities of condensate, containing the volatiles, possibly in a different proportion to the non-condensed gas, may be injected. Thus, acceptable repeatability may be difficult to achieve. Syringe heating was accomplished by drawing steam from boiling water through a glass syringe mantle. With the syringe mantle in place it was necessary to mark the plunger so that the volume injected was somewhat reproducible. To compensate for variations in injection volume, an internal standard was used. Propionitrile, which was used by several other workers (4, 9), was chosen.

With the equipment and procedures described, it was then necessary to determine the equilibration time or the best time at which to sample the headspace for the various foods. As noted, it would be convenient to follow the same procedures for all foods. Individual duplicate samples were prepared for each time studied by spiking 10 g slurry with 1 mL AN–PN solution. It was important to ensure that the added 1 mL solution was properly mixed by swirling to give a homogeneous mixture. Mixing peanut butter and standard was more difficult than for other foods. The time–response curves for AN, PN and their peak height ratios for honey butter, margarine, cold-pack cheese, and peanut butter are shown in Figure 2. For honey butter there is an initial increase in AN and PN peak heights as equilibrium is attained followed by a gradual decrease. The AN-PN peak height ratio decreases slowly over a 4 h period. For margarine, there is a slight variation in AN and PN peak heights which may be expected from slight differences in injection volume. The internal standard appears to compensate for this quite well as the ratio of peak heights is relatively constant. For cold-pack cheese and for peanut butter, however, the situation is quite different. The PN response appears to attain an equilibrium after 1 h for cheese and 2 h for peanut butter. The AN, however, for both cheese and peanut butter attains a maximum at about 1 h and decreases thereafter. The resultant AN-PN ratio for both foods also decreases. Thus, for both peanut butter, cold-pack cheese, and to some extent for honey butter,

there is significant loss of AN with time compared with the internal standard. This phenomenon is best explained by a decomposition or reaction of AN which removes it from the system. It has been reported that AN reacts in vitro and in vivo with proteinaceous material (13, 14). In Figure 2, the results appear to correspond roughly to the amounts of proteinaceous material in each sample. Peanut butter and cheese with the higher protein content exhibit the greater loss of AN. For all foods examined, an equilibration time of 2 h would appear adequate to attain equilibrium, based on PN. At this 2 h sampling time, however, the concentration of AN in peanut butter, cold-pack cheese, and to a lesser extent in honey butter is decreasing. Despite the decreasing AN in these foods at 2 h, reproducible linear calibration curves can be readily obtained. Therefore, one may assume that sample heating is being carried out in a reproducible fashion.

The variation in loss of AN after 2 h equilibration for each food (Figure 2) results in different AN headspace responses. These responses for 10 ppb AN as well as 96 ppb PN are shown in Table 1. For margarine and honey butter the AN response is about twice that of peanut butter and nearly 3 times that of cold-pack cheese. The response for PN, which is not lost during equilibration (see Figure 2), is similar for all foods, with the lowest PN response for the high lipid-containing margarine. Presumably, if AN was not reactive its headspace response would parallel that of PN for each particular food. The similarity of margarine and honey butter response (Table 1) for AN can be explained by a loss of AN in honey butter (see Figure 2), reducing the AN response to that of margarine.

To evaluate effectively the sample preparation and chromatographic techniques described earlier, repeatability at several stages of the analysis was investigated. First, repeatability of the headspace procedure was evaluated. A 1 mL standard solution containing AN and PN at 0.48 and 0.47 μg/mL, respectively, was added to each of 6 headspace vials each containing 2 g salt and 10 mL water (1% MEHQ). After 0.5 h equilibration at 100°C the 1 mL headspace samples were removed and injected onto the GC system, using the heated gas-tight syringe. Acceptable repeatability (6.9% CV) was obtained.

Next we evaluated repeatability with respect to the preparation of calibration curve points for each particular food. As described in the method, 1 mL AN-PN standard was added to each of 6 vials containing 10 g food-water-salt slurry to give AN at 20 ppb. The contents of the vial were carefully mixed to ensure dispersion of AN and PN. Again, acceptable repeatabilities of 7.5, 8.3, 7.3, and 10.2% for margarine, honey butter, cold-pack cheese, and peanut butter, respectively, were obtained. This indicated that the calibration curve points could be generated reproducibly.

Furthermore, to determine if AN was lost by volatilization during blending and/or transfer, cold honey butter-water-salt slurries were spiked in duplicate at 100 ppb in the blender and in individual headspace vials. Results from the blender samples averaged 5% higher. For cold-pack cheese at 100 ppb, the vial-spiked samples were 2% higher yet at 40 ppb the blender-spiked samples were 10% higher. Thus, it was concluded that no appreciable loss of AN by volatilization during sample preparation occurred.

Finally, the entire contents of several AN-based plastic containers were analyzed according to the method. For each of these foods, 6 replicates from various points in the blender's contents were taken. For honey butter at 8.5 ppb, cheese spread at 11.9 ppb, cold-pack cheese at 45 ppb, and peanut butter at 42.5 and 37.5 ppb, repeatabilities were 9.1, 5.9, 18.1, 15.2, and 10.6%, respectively. These results confirm the homogeneity of the food-water-salt blend.

These experiments ensure the repeatability of the overall scheme of analysis shown in Figure 3, which emphasizes the parallel treatment of the sample and standard curve (spiked) manipulations and analysis.

With all foods studied, AN generally exhibited a linear response with different sensitivities for different foods (Table 1). These different responses are to be expected considering the loss of AN by reaction or decomposition as shown in Figure 2, as well as expected different partition coefficients for different foods. The loss of AN in peanut butter and cheese, however, did not affect linearity.

For each food it was necessary to prepare a standard curve using food packaged in glass or non-AN-based plastic, e.g., polyethylene. For specialty foods, which were only available packaged in AN-based plastic, it was necessary to remove any migrant AN in preparing calibration curves. The AN was readily stripped from the sample by repeated evaporations of excess added water. Using this procedure, calibration curves for honey butter were readily prepared. With incomplete removal of AN, the small peak arising from any remaining AN could

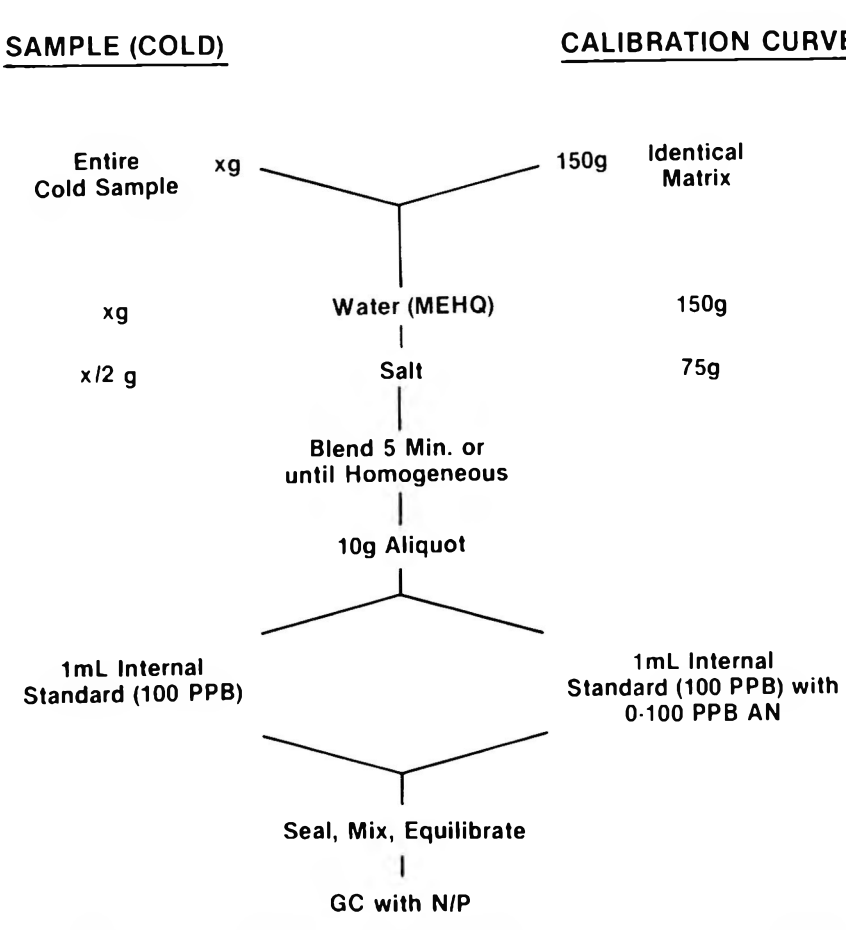

Figure 3. Analytical scheme emphasizing parallel manipulation for unknown and calibration samples.

be subtracted from all spiked AN peak heights before calculating peak height ratios.

With the exception of peanut butter, all foods examined were free of interferences (Figure 4). For peanut butter packaged in glass or non-AN-based plastic, a peak with a retention time corresponding to AN was present in all 7 different brands examined. This apparent AN ranged from 3 to 8 ppb in the samples examined. Because this peak occurred in a relatively narrow concentration range in all these samples, it was not believed to arise from AN contamination. It is believed to be an artifact produced during equilibration, because the interference continued to increase for up to 4 h heating. High resolution mass spectral investigation did show a peak corresponding to AN with approximate confirmation of level. Several other GC columns were unable to separate this apparent AN; other nitrogen-containing compounds were shown not to have similar retention times. Other late-eluting peaks, almost off-scale, also arose from peanut butter. After 2 injections, as described in the method, it was necessary to raise the column temperature to 200°C to rapidly elute these interferences before injecting 2 further samples.

Table 1. Headspace response characteristics for AN and PN in food [a]

Food	% FSD AN	% FSD PN
Margarine	24	52
Honey butter	26[b]	69
Cold-pack cheese	9	67
Peanut butter	13[b]	68

[a] AN and PN at 10 and 96 ppb and at 16 and 32 × 10^{-12} amp/mV, respectively, for 1 mL headspace (4% total) injection after 2 h equilibration.

[b] Corrected for blank.

The chromatograms shown in Figure 4 and data in Table 1 permit some estimate of the detection limits for each food. If the detection limit

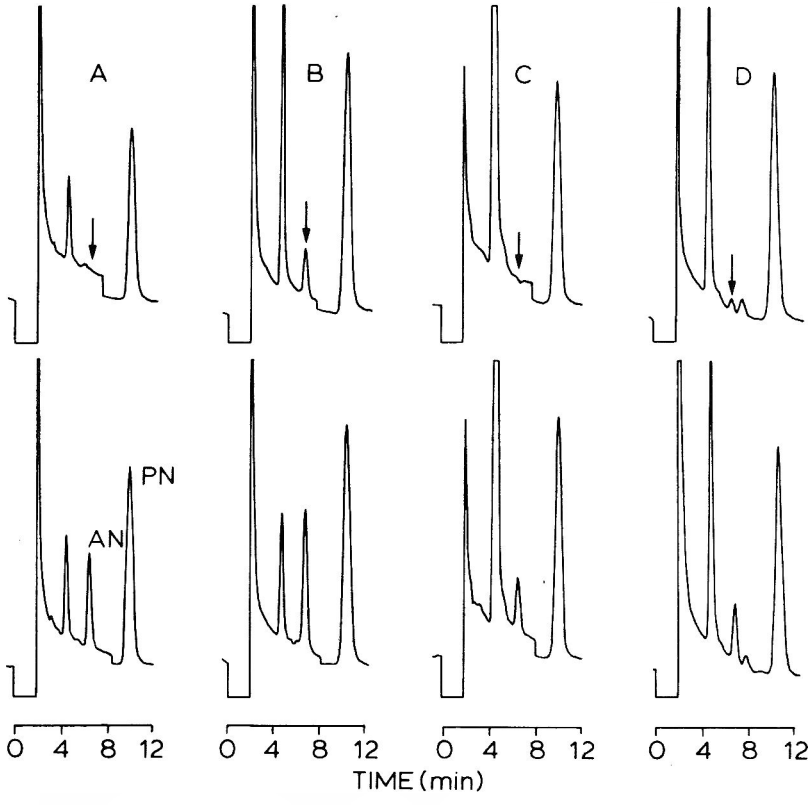

Figure 4. Typical blank (upper) and spiked (lower) chromatograms: A, margarine; B, honey butter after stripping, containing ca 5 ppb AN; C, cold-pack cheese; D, peanut butter. Arrow indicates retention time for AN in blank samples. AN spiked at 10 ppb (8×10^{-12} amp/mV) and PN at 96 ppb (16×10^{-12} amp/mV).

is defined as 10% FSD at 8×10^{-12} amp/mV, then 4–5 ppb AN in margarine or honey butter can be detected, whereas about 10 ppb would be needed to detect AN in cold-pack cheese. For peanut butter, although the blank values which ranged from 3 to 8 ppb could be justifiably subtracted in preparing calibration curves, one could not arbitrarily do this for actual AN-contaminated samples. Although the 13% FSD given in Table 1 would give a 7 or 8 ppb detection limit in peanut butter, a more realistic limit should be defined at 15 ppb, about twice the upper blank level.

Although our main interest lay in developing methodology for food, it was also desirable to briefly examine the AN-based plastic containers for AN levels. Before analysis of the residual AN in both food and container, it was necessary to identify the plastic to ensure that it was indeed AN-based. Dissolution in chloroform of a small piece or scraping from the container followed by evaporation of the solution or dispersion on a glass plate gave a film suitable for qualitative infrared analysis. The nitrile peak at 2260–2240 cm^{-1} was used to confirm the inclusion of acrylonitrile as a polymer constituent, and a comparison of the spectra with authentic reference spectra (15, 16) permitted tentative identification of the polymer.

The analysis of the AN-based resin for residual AN was carried out on a representative piece of the container. For circular tubs or jars, pie-shape segments were cut and for rectangular containers, quadrants were sampled. These samples were then cut into small pieces, weighed, and added to methyl isobutyl ketone (MIBK) in a suitable screw-cap vial. Dissolution or dispersion in the sealed vial was carried out overnight with mild shaking. For each gram of resin, 5 mL MIBK was used. An aliquot of the resulting dispersion was normally diluted in MIBK in a headspace vial before equilibration at 100°C for ½ h. When equilibrium was obtained, a gastight heated syringe was used to sample 1 mL of the headspace for GC analysis.

AN in the plastic was quantitated by compar-

Table 2. AN levels in some containers and contacted food

Food	Container material	AN level[b]			
		Sample[a]	Tub, ppm	Lid,[c] ppm	Food, ppb
Honey butter (natural)	ABS[d]	A	60	119	10.4, 15.8
		B	99.2	141	17.8, 22.4
		C	44.7	125	8.4, 22.6
Honey butter (cinnamon)	ABS	A	44.6	92.3	20.7, 18.4
		B	80.7	26	16.4, 15.0
		C	42.3	—[e]	16.5, 31.0
Cold-pack cheese	ABS	A	33.0		24.3, 23.8
		B	62.0		25.4, 29.4
		C	54.9		27.3, 31.5
Peanut butter	ABS-MM[f]	A	63.8		31.9, 38.1
		B	64.3		12.3, 11.5
		C	63.0		<10, 14.0
Soft butter spread	ABS	A	2.2		ND[g]
		B	1.7		ND
		C	1.7		ND
Creamed coconut	ABS	A	5.2		ND
		B	1.8		ND
		C	1.6		ND

[a] Different samples, same lot number.
[b] Single determination in plastic; duplicate determinations in food.
[c] Value given if AN-based plastic contacts food.
[d] ABS = acrylonitrile–butadiene–styrene polymer.
[e] Not analyzed.
[f] ABS-MM = acrylonitrile–butadiene–styrene methyl methacrylate polymer.
[g] ND = not detected; <2.5 ppb.

ing the peak height of the AN in the headspace of the plastic-MIBK dispersion to that of AN from a spiked MIBK solution containing no plastic. The small amount of plastic, usually 1–5 g, in the 10 mL of MIBK solution did not affect the equilibrium. Tentative results of the analysis of some AN-based plastic containers are compared with AN levels in the corresponding packaged food in Table 2. A rough correlation is evident. However, the time the plastic is in contact with the food as well as container parameters, such as wall thickness and area exposed to the food, would affect the ultimate level of AN in the food.

High resolution GC-MS confirmation of the identity and level of AN in several foods and containers was carried out by using a Varian Mat 311A mass spectrometer. AN was monitored at 53.0265 ± 0.0086 m/z and 52.0187 ± 0.0084 m/z in 2 separate determinations. The equilibration times, GC column, and headspace sampling was as for N-P detection. Quantitation was carried out by ratioing peak heights of samples and spiked blanks. The sensitivity of the GC-MS system operating at a resolution of 6200 was equivalent to the N-P detector for both direct and headspace injections.

Despite the varying responses of AN in various foods, the described procedure provides an approach to the analysis of AN in packaged foods where the AN would be expected to be inhomogeneously distributed. Further studies on the loss of AN during equilibration, and extension and application of the procedure to other foods has been carried out and will be reported later.

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WATER ACTIVITY

Filament Hygrometer for Water Activity Measurement: Interlaboratory Evaluation

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A method is presented for measuring water activity (a_w) of foods with a filament hygrometer. An interlaboratory evaluation carried out for the Nordic Committee on Food Analysis demonstrated that measurements can be made with a repeatability and reproducibility of 0.005 and 0.010 a_w, respectively. The critical parameters of a_w measurements are defined and discussed in relation to the accuracy and reproducibility required. The need for strictly standardized procedures for maintenance of the equipment, sample treatment, calibration, and measuring is emphasized.

The concept of water activity (a_w) was introduced in food microbiology by Scott (1) nearly 30 years ago. Since then, a significant amount of information has been accumulated about the relationships of water activity and microorganisms (2). This information, which includes spoilage organisms as well as pathogens, has resulted in wide acceptance of a_w as a well defined physico-chemical parameter controlling the microbial activity in foods.

In food control laboratories, the use of the concept of a_w was prevented for many years by lack of appropriate measuring methods and equipment. This situation has changed because several devices specifically developed for a_w measurements are now commercially available.

The various measuring methods suggested over the years can be grouped in 3 categories:

The first category comprises a wide range of commercial hygrometric devices designed for air humidity measurement in general. Considering the relation of a_w to microbial growth, which indicates that a_w differences of 0.01 are significant and also that a_w measurements of foods concentrate on high humidities, i.e., 70–95% RH, these devices appear not to be accurate enough.

The second category comprises relatively simple laboratory setups, such as various vapor pressure manometric techniques described by Lewicki et al. (3), the isopiestic method described by Fett (4) and Vos and Labuza (5), and the so-called proximity equilibration cell as described by McCune et al. (6) and modified by Lenart and Flink (7). These methods are not easy to deal with in a control laboratory, nor do they have sufficient capacity for routine measurements, so we do not find these methods applicable. However, it should be mentioned that the proximity equilibration cell method was published very recently and future investigations must show the applicability of this method.

The third category comprises commercial devices designed directly for determination of a_w for foods. Several comparative studies including such devices have been carried out (8–10). In these studies, instruments using immobilized salt solution sensors have played a central role and have generally been regarded as the most accurate equipment available. However, investigation by Jakobsen (10) and Rödel et al. (11) comparing this type of equipment with the simpler and significantly cheaper filament hygrometer (a_w-Wert-Messer Model 5803) based on the longitudinal changes in dimensions of a water-absorbing fiber, have concluded that both are accurate and convenient methods, allowing a_w determinations to be carried out routinely. Labuza et al. (12) and recent investigations by Rödel et al. (13) have also concluded that acceptable measurements can be carried out with the filament hygrometer.

The Nordic Committee on Food Analysis decided to carry out an interlaboratory study on the filament hygrometer. A main purpose of the study was to define and standardize the various parameters which are very critical for a_w measurements regardless of the method and equipment used.

METHOD

Principle

Water activity (a_w) is defined as $a_w = p/p_0$ where p is the equilibrium vapor pressure of the

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solution (the food product) and p_0 is the equilibrium vapor pressure of the solvent (water) at the same temperature. Then, $a_w \times 100$ equals the equilibrium relative humidity (ERH) produced by the food product in a closed system. ERH is measured by a hygrometer based on a polyamide filament showing measurable longitudinal changes for small changes in ERH at the high values that are of interest in food microbiology.

Areas of Application

Application of the method is limited to ERH values of 70–97%, corresponding to the a_w range of 0.70–0.97. Within this range the method can be used for ordinary food products: fruit, vegetable, meat, and fish products; confectionary and other products with high sugar contents; as well as products with high salt contents, etc.

Ethanol interacts with the filament and, therefore, the method cannot be used for products containing ethanol, e.g., wines. The possibilities for interactions with other volatiles must be kept in mind.

Apparatus and Reagents

(a) *Filament hygrometer.*—a_w-Wert-Messer, Model 5803, G. Lufft, Stuttgart, GFR. The sensing system is a stainless steel casing holding the water-sensitive filament and forming the lid of a stainless steel container. The container which holds the sample is 40–50 cm high and has a diameter of 80–100 mm. The filament is separated from the food sample by a perforated stainless steel plate. a_w can be read on a scale subdivided in units of 0.01 a_w. The final a_w of the sample is obtained from a standard curve.

During measurements, the hygrometers must be kept in an insulated box with a cover allowing the a_w to be read without opening the box. Measurements must be carried out at a constant temperature of 20 ± 1°C.

It is an indispensable requirement that the assembled apparatus is tightly sealed, so that no exchange of water vapor between the sample and the environment can take place.

The sample container must be kept absolutely clean. Any material allowed to remain in the container is likely to make the hygrometer inoperable. Extreme care must be taken that nothing is allowed to enter any part inside the hygrometer that cannot be cleaned. After cleaning, residues of detergents, etc., must not be left in the container. As far as possible, cleaning should be done with distilled water.

It must be ensured that the filament is not contaminated with sample material. Even the slightest contamination is expected to ruin the filament. The filament must also be protected from dust, and, therefore, the apparatus must be assembled and set aside in the insulating box when not in use. The filament should not be exposed to high RH values for long periods. Before the equipment is set aside, the meter should read less than 0.60 a_w.

(b) *Constant temperature cabinet.*—Cabinet must be capable of maintaining a constant temperature of 20 ± 1°C. The size of the cabinet should allow sufficient space for the hygrometer required as well as the samples that have to be equilibrated to the measuring temperature.

(c) *Reagents.*—The following salts (AR standard) are required: NaCl, KCl, $BaCl_2 \cdot 2H_2O$, KNO_3, and KH_2PO_4.

Sampling and Sample Preparation

As far as possible, the sample should be kept in the original packing until transferred to the measuring instrument. If the sample is transferred to another container, this container must be impermeable to water vapor and the volume should not exceed the sample unit by more than 10%.

It is essential that the samples are equilibrated for sufficient time to reach the measuring temperature. Even for smaller samples, the time required for equilibration amounts to several hours.

Solid samples should be comminuted before they are transferred to the measuring instrument.

If differences in a_w are expected within the same product, the sample should be taken from that part having the highest a_w. In some instances, it can be necessary to measure 2 separate samples, e.g., surface and center. For composite products, it must be decided from knowledge of the particular product whether the various components should be measured separately. For meat products in pieces, the samples must be taken from the lean meat.

Measuring Procedure

The sample volume shall amount to one-third the volume of the measuring chamber. As part of the standardization of the method, the sample volume must be standardized. The measuring container is closed as quickly as possible and transferred to the constant temperature cabinet. After exactly 3 h, the a_w of the sample is read. The reading is made with an accuracy of 0.005 a_w.

The final value obtained from the standard curve is reported to 2 decimal places.

After each measurement, the sensors must be exposed to a relative humidity ensuring that the meter reads less than 0.60 a_w before the next measurement.

Calibration and Production of Standard Curve

Measurements are carried out for the following saturated salt solutions with known a_w, at 20 ± 1°C:

Salt	a_w
NaCl	0.753
KCl	0.843
$BaCl_2 \cdot 2H_2O$	0.900
KNO_3	0.935
KH_2PO_4	0.952

The saturated salt solutions are produced by dissolving the salts in 30–40°C water with subsequent crystallization at room temperature. The saturated solutions are left for one week at room temperature before they are used.

Calibration is carried out by measuring the saturated $BaCl_2$ solution which shall result in a reading of 0.90. If this is not so, the meter is adjusted. The need for calibration is determined by the use of the hygrometer. However, it is recommended that calibration be done at least once a week.

The standard curve is produced from duplicate measurements of the 5 saturated salt solutions. The standard curve is constructed from the mean values by fitting the best straight line determined by regression analysis for the 5 points. The frequency required for making standard curves varies with the use of the hygrometer. It is recommended that a new curve be constructed at least once every month.

The repeatability and reproducibility of the method correspond to ±0.005 a_w and ± 0.010 a_w, respectively.

Interlaboratory Study

Each of 10 participating laboratories analyzed the following 10 food samples: tomato ketchup, tomato purée, blackcurrant jam, raspberry jam, bacon, 3 types of salami, ham, and marzipan. All samples were homogenized to eliminate sampling errors and packed in 12 oz tins. The tins were filled completely and hermetically sealed. The samples were sent to the participants in 2 series of 5 samples. Time elapsed between the series was 2 weeks. The participants were instructed that the unopened tins should be transferred to a refrigerator immediately after receipt. Before analysis, the tins should be kept overnight in a temperature-controlled cabinet, 20 ± 1°C, to equilibrate to the measuring temperature. The measuring equipment and procedure was in complete agreement with the method above. A standard curve was produced for each of the 2 series of samples. All samples were measured in duplicate. Data were reported on the form sent with the samples to the participants. All data, noncorrected and corrected values, as well as the standard curves produced for each sensor were reported.

Results and Discussion

All results reported from the 10 participating laboratories are shown in Table 1. According to a statistical evaluation of the results, it was decided not to reject any values reported. From the comments given by the participants and from the calibration check points, i.e., the standard curves reported, it also appears that the method suggested has been followed very strictly.

The statistics, calculated according to Youden and Steiner (14), are shown in Table 2. The s_b value, in general, is larger than s_r, which shows that, as expected, systematic error is the major reason for dispersion of results. This is also demonstrated by the calculated F-values (s_d^2/s_r^2). As a consequence of these systematic errors, the 95% confidence intervals are rather large, as seen from the table. However, considering the pooled values for repeatability (s_r) and reproducibility (s_d), they will be 0.005 and 0.010, respectively. The values, when compared with similar investigations (8, 9), are fully acceptable. This very satisfactory performance of the filament hygrometer has also been documented in comparative studies with immobilized salt solution sensors (10) where no significant differences were detected between the 2 types of instruments.

Among other factors, we suggest that the use of a standard curve in addition to the calibration recommended by the producer is the major explanation for the satisfactory performance of the hygrometer. According to the instruments from the producers, only one calibration check point, 0.90 (the saturated $BaCl_2$ solution), is required. However, in previous investigations (15), we found that the hygrometer is subject to an error which increases with the distance from the calibration point. The error varied from one set of hygrometers to the other but often amounted to 0.02 a_w. Such findings, which were confirmed

Table 1. a_w Values reported from participating laboratories

Sample	Laboratory									
	1	2	3	4	5	6	7	8	9	10
1	0.967	0.965	0.954	0.945	0.956	0.952	0.958	0.965	0.951	0.957
(tomato ketchup)	0.958	0.970	0.956	0.961	0.966	0.951	0.952	0.966	0.952	0.963
2	0.813	0.817	0.804	0.798	0.812	0.793	0.815	0.812	0.808	0.822
(marzipan)	0.808	0.815	0.808	0.818	0.817	0.809	0.800	0.810	0.798	0.812
3	0.740	0.752	0.747	0.734	0.755	0.738	0.749	0.742	0.748	0.752
(salami "farmer")	0.752	0.750	0.744	0.733	0.760	0.743	0.746	0.741	0.743	0.737
4	0.838	0.841	0.858	0.832	0.835	0.826	0.836	0.830	0.841	0.842
(salami 1)	0.840	0.838	0.864	0.834	0.839	0.839	0.834	0.833	0.845	0.843
5	0.966	0.966	0.956	0.963	0.983	0.974	0.946	0.956	0.962	0.957
(bacon)	0.954	0.970	0.956	0.961	0.987	0.970	0.952	0.957	0.962	0.945
6	0.800	0.798	0.814	0.789	0.807	0.820	0.806	0.801	0.796	0.800
(blackcurrant jam)	0.820	0.799	0.803	0.791	0.802	0.820	0.803	0.802	0.798	0.800
7	0.982	0.972	0.956	0.984	1.01	0.988	0.986	0.972	0.976	0.972
(tomato purée)	0.962	0.975	0.956	0.985	1.01	0.974	0.987	0.980	0.969	0.965
8	0.746	0.743	0.734	0.722	0.747	0.756	0.737	0.759	0.757	0.740
(salami 2)	0.740	0.741	0.750	0.726	0.748	0.758	0.739	0.754	0.757	0.730
9	0.940	0.921	0.926	0.936	0.971	0.942	0.931	0.931	0.954	0.937
(raspberry jam)	0.940	0.927	0.928	0.943	0.974	0.936	0.934	0.930	0.946	0.936
10	0.946	0.926	0.929	0.936	0.940	0.940	0.932	0.957	0.952	0.937
(ham)	0.940	0.930	0.924	0.940	0.948	0.932	0.937	0.960	0.942	0.936

Table 2. Statistical analysis of data shown in Table 1 [a]

Sample	$\bar{x}$	s_r	s_b	s_d	s_d^2/s_r^2	95% Confidence interval ($t \times 2s_d$)
1	0.959	0.005	0.005	0.006	1.4	0.959 ± 0.012
2	0.809	0.008	0.000	0.005	0.4	0.809 ± 0.010
3	0.746	0.005	0.006	0.007	2.0	0.746 ± 0.015
4	0.840	0.004	0.009	0.009	5.1***	0.840 ± 0.019
5	0.963	0.004	0.010	0.010	6.3***	0.963 ± 0.021
6	0.804	0.005	0.007	0.008	2.6*	0.804 ± 0.017
7	0.978	0.006	0.013	0.014	5.4***	0.978 ± 0.029
8	0.744	0.005	0.010	0.011	4.8**	0.744 ± 0.013
9	0.939	0.003	0.014	0.014	21.8***	0.939 ± 0.029
10	0.939	0.004	0.009	0.009	5.1***	0.939 ± 0.019

[a] n = number of single determinations = 20 for all samples.
$\bar{x}$ = mean of single determinations.
s_r = precision (repeatability).
s_b = systematic error.
s_d = total error (reproducibility).
*, **, *** 90, 95, and 99% probability level for systematic errors.

in the present study, are the main reason for considering the use of a standard curve as an indispensible requirement.

Contrary to other methods for calibration or check of instruments or devices for measurement of a_w, e.g., those described by Stoloff (9), the present method uses a fixed measuring time of 3 h instead of a measuring time defined as an approach to equilibration; in the latter technique, readings may be carried out after 15, 30, 60, and 120 min and continued at 60 min intervals, if necessary, until 2 consecutive readings vary by less than 0.01 a_w. According to earlier investigations of the filament hygrometer as well as other methods for measuring a_w (10), we find that a fixed measuring time represents a higher degree of standardization and also makes a_w measurements more convenient to handle in the daily routine of a control laboratory. Such a measuring procedure obviously must be seen in relation to an otherwise highly standardized method including only smaller variations in sample volumes.

In the present investigation, the measuring temperature was 20°C, the temperature at which the hygrometer is calibrated by the producer. The application of another measuring temperature, e.g., 25°C, was avoided because it would have introduced an extra correction factor. This could have been a disturbing element in the study which, in particular, was intended to evaluate the applicability of the hygrometer. From the promising results obtained and from previous experience with the apparatus (10), we see no objection to carrying out calibration and measurements at other temperatures, e.g., 25°C.

The use of saturated salt solution or salt slushes as references is widely accepted. More recent investigators (9, 16) seem to prefer slushes to saturated solutions. In comparing the two, we found no detectable difference in measurements carried out with a filament hygrometer and measurements carried out with an immobilized salt sensor (unpublished results). The present, as well as previous investigations (10, 15) using saturated salt solutions prepared as described in the method support this finding. However, for standardization, one method and one set of reference values at 25 or 20°C, or even at both temperatures, should be agreed on internationally. More recent publications seem to concentrate on the reference values compiled by Greenspan (16). However, microbiological data, such as a_w limits for growth, are generally established on experiments using other data, e.g., those produced by Stokes and Robinson (17), as reference values. As existing as well as future

a_w regulations will predominantly refer to limits for growth, this should be considered when selecting a set of reference values.

Apart from the necessity for standardization of measuring temperatures and reference values, several other critical parameters are maintenance of equipment, prevention of the hysteresis effect, temperature equilibration of samples, constant measuring temperatures, etc., are prerequisites for acceptable a_w measurements, as pointed out in the present investigation. Provided these parameters are considered, it appears that water activity measurements can be made with an accuracy and reproducibility of ± 0.010 a_w.

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METALS AND OTHER ELEMENTS

Release of Lead and Cadmium: Comparison of Two Hot Leach Methods with a Room Temperature Method, Using Specially Glazed Ceramic Ware

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Oven and hot plate leach methods were compared with the official AOAC room temperature method for leaching Pb and Cd from specially glazed ceramic cookware. The hot leach methods released from 7 to 11 times more Pb and from 6 to 10 times more Cd after 24 h than did the AOAC procedure. Both hot leach methods specify a 2 h treatment with boiling 4% acetic acid, followed by 22 h of cooling at room temperature. The results show that essentially all of the Pb and Cd are released within 2 h, suggesting that the total contact time can be limited to 2 h to save laboratory space and time. The hot plate method is less difficult to perform and results in a continuous refluxing action. For these reasons, it is proposed as the hot leach method of choice.

The official AOAC room temperature (RT) leach method (1) for the testing of ceramic foodware for Pb and Cd is identical to that of the American Society for Testing and Materials (2). The RT method has achieved international recognition since it has been adopted by the International Standards Organization (3), and is now being used by a number of other countries (4, 5) besides the United States. The RT test requires that the ware be filled with 4% acetic acid solution and allowed to stand at RT for 24 h. The quantity of Pb and/or Cd released from the glazed ceramic ware is then determined by atomic absorption spectrophotometry (AAS).

The quantity of Pb and Cd released by boiling 4% acetic acid may exceed by several times, depending on the nature of the surface, the quantity released from the same surface at RT (6–8). Furthermore, foods heated or cooked in contact with such metal-releasing surfaces can become contaminated (9–11). Thus, it is important to investigate hot leach methods.

Two hot leach methods, an oven test used in the United Kingdom (12) and a hot plate test that was proposed at a 1979 meeting of the World Health Organization (13), were compared with the AOAC RT leach method (1).

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METHODS

Principal

The British Standard 4860 part 2 (oven test) (12) is intended to apply to cooking ware, that is, glazed pottery articles that are heated in one of several ways depending on the intended use.

The hot plate test (13) is applied to any cookware in which food is heated during preparation. This includes glazed china, porcelain enameled ware, and glass or glass ceramics, but excludes items designed for microwave cooking.

In both hot leach methods, Pb and Cd are determined by AAS; values are reported as parts per million in the leach solution.

Apparatus

Glass and plastic ware used to prepare, transfer, or store analytical and leach solutions must be soaked overnight in concentrated HNO_3–water (2 + 3) followed by a water rinse before use.

(a) *Atomic absorption spectrophotometer.*—Model 403 (Perkin-Elmer Corp., Norwalk, CT 06856); with Pb and Cd hollow cathode lamps, and a burner for C_2H_2 and air. The sensitivity of this instrument was determined from uncorrected standard solutions, and was found to be:

For Pb: (n = 21) absorbance/ppm Pb = 0.0066 ± 0.0007 (standard deviation).

For Cd: (n = 20) absorbance/ppm Cd = 0.1108 ± 0.0109.

(b) *Hot plates.*—Model HP-A1915B (Thermoline Corp., Dubuque, IA 52001).

(c) *Oven.*—Model 601 (Hotpack Corp., Philadelphia, PA 19154).

Reagents

Use deionized, distilled water.

(a) *Acetic acid.*—Glacial (ACS) (J.T. Baker Chemical Co., Phillipsburg, NJ 08865). Mix with water (1 + 24) as required for leaching by AOAC or British oven test.

(b) *Detergent wash.*—Add 15 g alkaline detergent (e.g., Calgonite, Calgon Corp., Pittsburgh, PA 15230) to 1 gal. (3.8 L) water.

(c) *Lead standards.—Stock solution.*—1 mg/mL. Cut lead shot (Baker Analyzed, J.T. Baker Chemical Co.) into pieces with a single-edge razor and dissolve 1.000 g in 10 mL HNO_3. Dilute to 1 L with water. *Working solutions.*—Dilute 0.0, 0.025, 0.050, 0.100, 0.250, 0.500, and 1.00 mL stock solution to 50 mL with 4% acetic acid (0.00, 0.50, 1.00, 2.00, 5.00, 10.00, and 20.0 μg Pb/mL).

(d) *Cadmium standards.—Stock solution.*—1 mg/mL. Cut thin tails from Cd metal shot (Cominco High Purity Metals, Allied Chemical Co., Morristown, NJ 07960) and dissolve 1.000 g in 10 mL HCl. Dilute to 1 L with water. *Working solutions.*—Dilute 0.0 and 0.025 mL stock solution to 100 mL with 4% acetic acid, and 0.025, 0.050, and 0.10 mL stock solution to 50 mL with 4% acetic acid (0.0, 0.25, 0.50, 1.00, and 2.00 μg Cd/mL).

Ware Samples

Six pieces of ware were assigned to each of the 3 test methods. The ware, not a commercial product, had been prepared with a special red Pb- and Cd-releasing glaze on the food-contact surface of a 103 mm id earthenware casserole, which had a ceramic cover with the same glaze. The depth of the leach solution used ranged from 35 to 36.5 mm for both of the hot leach methods, and from 43.5 to 45 mm for the AOAC method.

If no covers are provided, then the ware subjected to hot leaching must have substitute covers of clean, acid-washed borosilicate glass to prevent excessive evaporation.

Volume of Leach Solutions

Both hot leach methods specify that the volume of the leach solution should be two-thirds of the total volume of the ware, which is defined as the volume required to fill the ware to overflowing or to the cover rest. The fill volume to the cover rest, which was used in these tests, was found to be 420 mL in 2 pieces of ware; thus the hot leach volume was 280 mL.

The AOAC method specifies filling within 6–7 mm (1/4 in.) of overflowing, or in this case, the cover rest. This volume was 350 mL.

When the hot leach fill volume of the ware is being determined, marked glass rods to use as dip-sticks can be prepared. These rods indicate the proper depth of the leach solution and make it easy to restore solution lost by evaporation during heating.

Cleaning the Ware

All internal surfaces of the ware were cleaned by washing with the detergent wash and a pad of absorbent cotton. These surfaces must be free of grease and dirt, which could prevent proper wetting by the leach solutions, or in the case of hot leaching, by the vapors. The clean ware was rinsed with water and allowed to drain dry.

Oven Hot Leach Method

Six of the clean, dry casseroles, along with their covers, were placed in a 120°C oven. (A forced-draft oven is suggested in the method, but a convection oven was used for this work.) While the ware was being warmed to reduce the thermal shock of adding the boiling leach solution, enough 4% acetic acid solution to fill the ware was heated to boiling separately. After the ware was heated, the required quantity of boiling acid was added to each piece and the filled ware was covered and returned to the 120°C oven for 2 h.

When the ware was removed from the oven, solvent was restored to the hot samples by adding fresh RT 4% acetic acid to the mark on the dip-stick. Following the method, the ware with the leach solution restored would have been covered, set aside, and allowed to cool to RT and to stand for an additional 22 h, thus making the contact time 24 h before the solution was assayed for Pb and Cd. In this experiment, however, the restored leach solutions were all sampled after 2 h as well as at 24 h. The quantity of solution required for the 2 h hot test samples ranged from 0.5 to 1.0 mL, thus causing no significant deviation from the proper leach volume requirements of the test.

Hot Plate Method

The RT ware, containing enough distilled water to provide the required quantity of leach solution *after* the acetic acid was added, was heated until the water came to a slow boil or a simmer. Then enough glacial acetic acid was added to give a 4% solution, and the timing of the 2 h hot contact period began. In this case, 270 mL RT water was heated to boiling before 10.7 mL glacial acetic acid was added to each casserole. The hot plates were adjusted to maintain a slow boiling in the covered samples. The ware was removed from the heat after 2 h. The solvent level was restored with 4% acetic acid, and

the 2 h sample of 0.5–1.0 mL was taken. The covered ware was allowed to stand at RT for 22 h, and the RT solution was then sampled again.

AOAC RT Method (1)

The ware was filled with 4% acetic acid, and the solution was sampled after 2 and 24 h at RT.

Results

Table 1 shows that the average concentration of Pb found in the 24 h samples by the oven method was about 7% more than that found in the 2 h sample studied. The hot plate method solutions also showed that the increase in the average Pb content for the 24 h sample was about 5% more than that present in the 2 h sample. On the other hand, with the AOAC RT method, the average Pb content increased by 106% on standing at RT for 22 h. The coefficient of variation (CV) of all of the individual readings ranged from about 6 to 12%.

The average 24 h Cd content was less than the 2 h value for both hot leach systems (Table 1). The reduction of Cd in the oven test samples was about 10%, while a 16% decrease was found in the hot plate test samples. Conversely, the Cd concentration increased in the AOAC RT 24 h leach solution by about 107%.

The average quantity of Pb in the 2 h hot plate test samples was about 58% more, and the 24 h sample was about 54% more, than the corresponding oven test values. Similarly, the hot plate test release of Cd exceeded the average oven test values by about 65% for the 2 h and by about 54% for the 24 h sample.

Both of the hot leach results far exceeded the average values of Pb and Cd released by the AOAC RT method. The average Pb concentration in the 2 h oven sample was about 7 times that of the 24 h AOAC RT Pb content, while the hot plate sample was about 11 times that of the AOAC RT content. The corresponding comparison for Cd showed that the 2 h oven samples contained about 7 times and the hot plate samples about 12 times the quantity of Cd found in the 24 h AOAC RT solution.

Table 1 also includes a calculation of the quantity of metal released in mg/sq. dm to show the extractive power of the various leach solutions based on a unit area of ware wet by the leach solution as the common basis for comparison. Paired *t*-tests were used to determine if differences existed between the 2 and 24 h test results. Significant differences were found at the 0.99 ($P \leq 0.01$) level for the hot plate test Cd, the oven test Pb and Cd, and the AOAC RT Pb and Cd. Results for Pb released during the hot plate test were significantly different at the 0.975 ($P \leq 0.025$) level.

Table 1. Pb and Cd concentration (ppm) of leach solutions determined by using oven, hot plate, and AOAC methods

	Oven		Hot plate		AOAC	
Ware	2 h	24 h	2 h	24 h	2 h	24 h
			Lead			
1	94.4	105.9	171.3	188.3	5.8	14.9
2	102.9	110.1	150.5	160.3	7.0	15.1
3	93.7	100.2	178.2	182.6	8.1	16.8
4	96.0	103.5	156.7	163.6	7.7	11.4
5	110.6	114.2	166.7	163.6	7.4	15.7
6	111.4	118.3	138.3	148.0	7.1	15.1
Av., ppm	101.52	108.69	160.27	167.72	7.19	14.82
SD, ppm	8.04	6.80	14.67	14.99	0.81	1.81
CV, %	7.9	6.3	9.2	8.9	11.3	12.2
24 h rel. to 2 h (% change)	+7		+5		+106	
Metal[a]	14.3	15.3	22.6	23.6	1.1	2.3
			Cadmium			
1	36.5	34.9	75.9	66.4	2.7	5.3
2	41.6	36.7	63.8	53.3	2.8	5.7
3	40.3	37.8	76.9	64.2	3.1	6.5
4	36.2	34.8	63.5	53.8	2.9	5.9
5	44.2	38.1	65.5	53.2	2.7	5.7
6	46.1	39.0	58.0	49.7	2.5	5.5
Av., ppm	40.81	36.89	67.24	56.76	2.78	5.76
SD, ppm	4.00	1.74	7.50	6.80	0.21	0.42
CV, %	9.8	4.7	11.2	12.0	7.6	7.3
24 h rel. to 2 h (% change)	−10		−16		+107	
Metal[a]	5.7	5.2	9.5	8.0	0.4	0.9

[a] Metal released in mg Pb or Cd/sq. dm of area wet by leach solution.

The loss of leach acid was measurable only for the hot leach procedures. The lost volume was restored by adding 4% acetic acid to the hot solutions after the 2 h heating period. The oven method required an average of 73.0 mL acetic acid (standard deviation (SD) 25.7) and the hot plate method required an average of 73.3 mL (SD 11.8) for the 6 pieces of ware tested.

The precision of the use of dip-sticks was determined by filling the 12 casseroles that had previously been used in the 2 hot leach tests with RT distilled water. This volume ranged from 278 to 290 mL, average 284.5 mL (SD 4.27, CV 1.5%). This shows the excellent precision of the dip-stick technique; however, the 1.6% error of the average value relative to the nominal volume of 280 mL probably depends on chance, that is,

on the size of the single piece of ware initially selected as being representative when the dipstick was prepared.

Discussion

The pattern of Pb release found in this study is similar to that reported by Beckman and Sark (14) although their boiling leach conditions were different from those described here. They found that the hot leach Pb concentration exceeded the 24 h RT values by a factor of 10. These workers also extended the RT contact to 2 months. After 1 month the Pb concentration was about the same as that found in the short-term boiled sample, while after 2 months the Pb concentration in the RT solution exceeded the initial hot leach concentration by a factor of about 2.

In this experiment, the average Pb content of the oven leach solution increased by 2000 μg, the average of the hot plate samples by 2100 μg, and the RT leach average increased by 2700 μg during the 22 h that all of the samples stood at RT after the initial 2 h of heating or of RT contact. These values are remarkably similar, when one considers that the quantity of Pb that had already been released by the glaze in the first 2 h was greatly different. The initial quantity of Pb released ranged from an average total of 28 000 μg into the oven leach samples and 45 000 μg into the hot plate samples, but only an average of 2500 μg Pb was released into the RT samples.

The experimental results all appear to support the concept that the Pb release from a glaze is a diffusion limited, ion exchange reaction. Such a Pb release mechanism could explain the tendency of the Pb concentration to increase with time of contact or increased temperature to the limit so that all of the soluble Pb (16) is eventually removed from the glaze. Furthermore, such a mechanism would tend not to be easily reversible, thus forcing the released Pb to remain in solution.

The Cd concentration in the AOAC RT leach solution continued to increase between the 2 and 24 h samplings. Beckman and Sark (14) also studied Cd release. Their data show that equilibrium for Cd concentration is apparently reached after only about 3 weeks at RT. One possible reason for the more rapidly achieved equilibrium of Cd ions (and one which is apparently supported by the observations in this study, which showed that the Cd concentration fell from 10 to 16% in both hot tests between the 2 and 24 h samplings) is that the colored Cd salts, along with CdO, are not part of the glaze matrix but are merely suspended in it as a separate crystalline solid (16, 17). The Cd ions could then dissolve out of the glaze to increase the solution concentration or crystallize out into the glaze matrix again to reduce it, depending on the Cd solubility in the leach acid. This system does not depend on the slow release of ions from an ion exchange matrix nor does it prevent the loss of ions already in solution by closing off the precipitation sites, as is proposed for Pb.

The increased release of Pb and Cd from the hot plate test solutions compared to the oven test solutions may result from a greater condensation on and reflux from the internal portions of the ware that are not wet directly by the hot solution. This greater reflux is expected in the hot plate test because these samples are heated at the bottom in an approximately RT ambient. This is in contrast to the oven method in which the samples are heated in a 120°C oven. At this temperature, the oven ambient is about 20°C higher than the boiling point of the 4% acetic acid leach solution. Such high ambient temperatures should not permit condensation and reflux to take place to any great extent. Consequently, the leaching by the oven technique may be limited largely to that glaze actually wet by the boiling acid, while the enhanced reflux possible with the hot plate method may allow a partial sampling of the entire inner surface of the ware.

The difference between 2 and 24 h average results, as shown by 6 paired *t*-tests, suggests that the full 24 h regimen may be required by the 2 hot leach methods, and that this possibly should be applied as directed by the methods (12, 13). However, it has also been shown by analogy that the 2 h heating and the 22 h cooling do not make all of the labile Pb available for measurement (18). Conversely, the concentration of Cd fell during the 22 h of standing after heating. This decrease appears to be an artifact of the leach solution in which the Cd ions are dissolved, for if a food substance were cooked or baked in such Cd-releasing ware as these test casseroles, then the released Cd could form a stable complex with the food, such as the element does in oysters (19) in nature, and in this way no longer be available for precipitation back into the glaze. If this complex is formed, the cooked food could contain all of the Cd released, and this may be much greater than the Cd remaining dissolved in the acid after 24 h of contact with the glaze.

Conclusions

A 2 h hot leach approximates an average cooking period, with the advantage over the full

24 h contact time that is specified by the methods, of conserving laboratory space and time.

The hot plate method appears to be more efficient than the oven method in extracting Pb and Cd. The hot plate method is also technically less demanding than the oven method because boiling acetic acid solutions need not be handled. For these reasons the hot plate leach, modified to 2 h of contact, is proposed as a hot leach method. The hot plate method has been collaboratively studied and has been adopted interim official first action (20).

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Improved Apparatus for Rapid Mercury Determination by Cold Vapor Atomic Absorption Spectrometry

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A glass reaction vessel is described and the convenience and simplicity of its use in cold vapor atomic absorption spectrometric determination of mercury are pointed out. The vessel is easy to construct and requires only 15 s for collection of the analytical signal. It provides for continuous flow of carrier gas, and contains a septum-covered sample injection port, a buret for rapid introduction of reagent, and a drain stopcock. Using this apparatus, the relative standard deviation is 2.9% at the 40 ng Hg/mL level. The detection limit is 1 ng/mL. Applicability of the apparatus is proved by analysis of 3 commercial waste water quality control samples.

The literature on atomic absorption spectrometric determination of mercury atomized by chemical means is abundant. Since the first reports by Poluektov and coworkers in 1964 (1) and by Hatch and Ott in 1968 (2), describing the sensitivity afforded by evolving Hg vapor by means of a strong reducing solution, hundreds of reports have described applications and/or improvements of the technique.

The apparatus used for the cold-vapor Hg technique (method 245.1) approved by the Environmental Protection Agency (3) makes for a considerably slower and more laborious analysis compared with the one proposed in the present paper. The former technique involves preparing the Hg-containing samples for analysis in a series of simple 300 mL BOD bottles. Following digestion, a sample is treated with a strong reducing agent and the bottle is quickly attached to an aeration apparatus in which a pump cycles air through a closed system consisting of the BOD bottle, drying tube, and absorption cell positioned in the light path of the atomic absorption spectrophotometer. It takes approximately 1 min for the recorder pen to reach a maximum and level off. At that time, a bypass valve is opened and aeration is continued until all Hg has been flushed from the sample and absorbed in a waste solution. The stopper and the bubbler are then removed from the BOD bottle and the next sample in another BOD bottle is treated with reducing agent and quickly attached in position.

With the proposed apparatus, by contrast, the same reaction vessel continues to be used for all samples, with no rinsing required between runs, the analytical signal is obtained in about 15 s, the waste Hg is eliminated in a few seconds, and the results are reproducible and accurate.

The proposed apparatus has already been used to carry out several gas phase molecular spectrometric analyses. These include the determination of iodide and bromide by evolution of I_2 and Br_2 (4), sulfite by evolution of SO_2 (5), sulfide by evolution of H_2S (6), nitrite by evolution of NOCl (7), ammonium (8) and cyanide (9) by evolution of NH_3.

Experimental

Instrumentation and Reagents

Absorbance measurements were made on the Perkin-Elmer Model 373 atomic absorption spectrophotometer and readout was presented on the Perkin-Elmer Model 023 recorder. The 253.7 nm Hg line generated by the Perkin-Elmer Intensitron hollow cathode lamp was used with a slit setting of 0.7 nm. The burner head was removed and replaced with a T-shape open-ended glass tube, 165 mm long and 12 mm in diameter, which is supported by a holder fitting into the neck of the burner. (A quartz-windowed flow-through cell can also conveniently be used.) The carrier gas plus Hg vapor enter the absorption cell at the middle of the tube, flow toward the ends, and are exhausted into the hood. The position of the glass tube is optimized to allow the excitation beam to pass through its long axis.

In connection with the reaction vessel described in the next section, the following equipment was also used: Injections were made using Becton-Dickinson 5 mL disposable plastic syringes equipped with B-D 22 gage 1.5 in. disposable needles. The flow of compressed air was measured by means of a Fisher Lab Crest Century flowmeter.

Reagent grade chemicals and deionized water were used throughout. Nitric acid was Baker InstraAnalyzed for trace metal analysis. The

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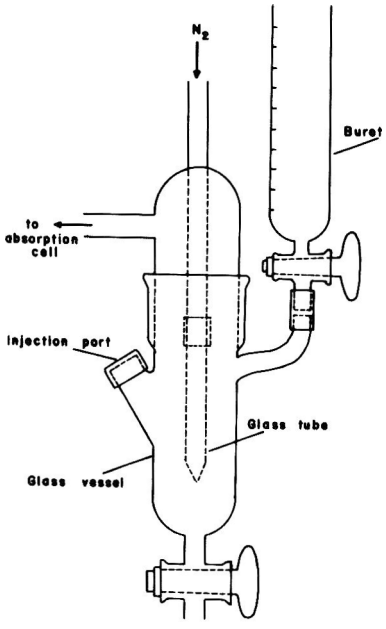

Figure 1. Reaction vessel.

1000 μg/mL Banko mercury reference standard served as the standard stock solution. The reducing solution consisted of 26.3 g $SnCl_2 \cdot 2H_2O$ diluted to 250 mL with 0.5N H_2SO_4.

Reaction Vessel and Procedure

The reaction vessel proposed for a speedy and convenient evolution of Hg vapor from solutions is illustrated in Figure 1. The glass vessel has a generally cylindrical shape and a total internal volume of about 86 mL. (The exact size and diameter of the vessel do affect the intensity of the recorded absorbance signals. No particular effort was made here to discover the optimum dimensions.) The carrier gas, after passing through a flowmeter, enters the reaction vessel through the plain glass tip (such as that from a medicine dropper). The gas is allowed to flow continuously and is not turned off until the end of the experiment. The carrier gas stream leaves the reaction vessel via a sidearm which is connected by a short piece of Tygon tubing to a T-shape absorption cell positioned in the light path of the spectrophotometer. The reaction vessel is connected to a 25 mL buret by means of a side-arm and a short sleeve of Tygon. The injection port is covered by a rubber septum. The reaction vessel is equipped with a stopcock at the bottom.

A 3.00 mL aliquot of the Sn(II) reducing solution is delivered from the buret into the reaction vessel. The tip of the carrier gas tube is submerged by the liquid and the continuously flowing gas provides vigorous agitation of the solution. The recorder is turned on and a baseline is established. The aliquot of Hg-containing solution is introduced with the aid of a syringe through the rubber septum. To ensure optimum precision, it is important to carry out the injection at as rapid and consistent a rate as possible, directing the needle at approximately the same angle every time. The carrier gas causes rapid mixing of the injected sample with the reducing solution. As the evolved Hg vapor is carried through the absorption cell, it gives rise to a transient signal which reaches a maximum in a few seconds and then decays back to the baseline much more slowly. The significant advantage of equipping the reaction vessel with a stopcock at the bottom is that the contents can be drained as soon as the recorded signal has passed its maximum. The waste is drained into a large beaker containing some $KMnO_4$ solution. The fact that some of the Hg is removed from the reaction vessel without having had a chance to become evolved into the gas phase and to pass through the absorption cell is irrelevant because it is the peak height rather than the peak area of the recorded signals which is measured. Measuring peak height is simple and fast, and this measurement remains proportional to the concentration of Hg in the sample solutions over a substantial concentration range, as the data given below demonstrate.

As soon as the recorded absorbance signal has passed its maximum (about 15 s after injection, see data below), the recorder is turned to standby position, the reaction mixture is drained, the stopcock is closed, a fresh aliquot of reducing solution is measured in, the recorder is turned back on, and as soon as a small portion of the flat baseline has been traced out, the system is ready to receive the next injection. There is no need to rinse the reaction vessel between runs. Any residual Hg would be carried away by the continuously flowing carrier gas while the cell is being refilled with the fresh aliquot of reducing solution. In addition, any residual Hg would be easily detectable by a sloping baseline. (No residual Hg effects were actually ever observed in this work.)

Results and Discussion

Optimizing Volume of Reducing Agent

The volume of reducing solution measured into the reaction vessel from the sidearm buret

Table 1. Effect of volume of reducing solution on absorbance intensity

Volume, mL	Absorbance, peak ht
1.00	0.146 ± 0.004
2.00	0.139 ± 0.003
3.00	0.128 ± 0.003
5.00	0.123 ± 0.004
7.00	0.110 ± 0.006
10.00	0.102 ± 0.005

Table 2. Effect of carrier gas flow rate on absorbance signal

Air flow rate, L/min	Peak time, s	Absorbance, peak ht
0.2	46	0.133 ± 0.004
0.6	15	0.129 ± 0.006
1.0	9	0.124 ± 0.007
1.5	8	0.110 ± 0.005

was varied from 1.00 to 10.00 mL. Repeated 5.00 mL injections of the 20.0 ng Hg/mL standard were made at each volume. The results are presented in Table 1. The intensity of the absorption signal decreases as the volume of the reducing solution is increased. However, because of the shape of the reaction vessel, volumes below 3 mL do not permit efficient mixing of the injected sample with the reducing solution and the recorded absorbance signals acquire a jagged, instead of a smooth, shape. A volume of 3.00 mL was selected as optimum.

Optimizing Carrier Gas Flow Rate

Keeping the volume of reducing solution at 3.00 mL and making repeated 5.00 mL injections of the 20.0 ng Hg/mL standard, the flow rate of air used to carry the evolved Hg vapor from the reaction vessel to the absorption cell was varied. The results are presented in Table 2. As has been observed with other applications of the proposed reaction vessel, the effect of the flow rate of the carrier gas on the intensity of the absorbance signal is slight, decreasing only from 0.133 to 0.110 as the flow rate was increased from 0.2 to 1.5 L/min.

Whereas the effect of carrier gas flow rate on signal intensity is not very significant, its effect on the speed with which the absorption signal arises is quite significant. Peak time indicated in Table 2 is the time elapsed between the instant when ½ of the sample aliquot has been injected and the emergence of the sharp maximum of the recorded peak. At the 0.2 L/min flow rate, the recorded signal is very broad and extends analysis time unnecessarily. At flow rates of 1.0 L/min and above, the absorption peak begins to emerge on the recorder before the injection of the entire 5.00 mL sample aliquot can be completed. A flow rate of 0.6 L/min offers near optimum signal intensity and convenient peak time.

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Dynamic Range

Five mL aliquots of Hg standards ranging from 2.0 to 80.0 ng/mL and a blank were injected several times into fresh 3.00 mL aliquots of reducing solution with a continuous air flow rate of 0.6 L/min. When the height of the recorded peak above the baseline was measured, a linear calibration curve was obtained up to 40.0 ng/mL. The line was very slightly curved up to 60.0 ng/mL and appreciably curved thereafter.

Precision and Detection Limit

Ten replicate injections of the several standards were made under the usual optimum conditions to test the reproducibility of the technique. The 4.00 ng Hg/mL standard yielded an average absorbance of 0.0233 (with an average deviation from the mean of 0.0024 and a relative standard deviation of 14%). The 20.0 ng/mL standard yielded an average absorbance of 0.116 (with an average deviation from the mean of 0.006 and a relative standard deviation of 8.0%). The 40.0 ng/mL standard gave an average absorbance of 0.226 (with an average deviation from the mean of 0.005 and a relative standard deviation of 2.9%).

Defining the detection limit as that concentration which gives a signal twice the size of standard deviation from the mean of repeated injections of the 4.00 ng Hg/mL standard, yields a detection limit of 1.0 ng/mL for the technique.

Accuracy

To show the accuracy of the technique, 3 waste water quality control samples supplied by the Environmental Resource Associates (ERA, 120 E. Sauk Trail, South Chicago Heights, IL 60411) were analyzed. Control samples No. 8008, 9201, and 2767 came in the form of concentrates and were diluted 5.00 mL to 500 mL with deionized water and 5 mL of concentrated ultrapure HNO_3. An appropriate acid blank was also prepared.

Table 3. Analysis of commercial waste water standards (ng Hg/mL)

ERA standard	"True" Hg concn	$\pm 2\delta$ range	Proposed method
8008	1.4	1–3	1.9 ± 0.4
9201	4.3	3–5.6	4.7 ± 0.3
2767	21.0	11–32	12.3 ± 0.4

Each sample and a series of standards were injected 4 times and the signals were averaged. The results are given in Table 3. The "true" values supplied by the ERA represent the results of analysis of the given concentrates by a series of independent laboratories and the $\pm 2\delta$ range represents the spread of the answers supplied by these laboratories. In all 3 cases, the results obtained by the proposed technique fell within the indicated ranges, and in 2 of the cases, the results were very close to the "true" averages.

The proposed technique for the cold vapor atomic absorption determination of mercury combines accuracy and sensitivity with improved speed and convenience.

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Electron Capture Gas-Liquid Chromatographic Determination of Methyl Mercury in Fish and Shellfish: Collaborative Study

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A method for determining methyl mercury in fish and shellfish was collaboratively studied in 8 laboratories. Methyl mercury is isolated from acetone-washed, homogenized tissue by adding hydrochloric acid and extracting into benzene the methyl mercuric chloride that is formed. The benzene extract is concentrated and analyzed for methyl mercuric chloride by electron capture gas-liquid chromatography on 5% DEGS-PS treated with inorganic mercuric chloride solution. The quantitation limit for the method is 0.05 μg Hg/g. Each collaborator determined methyl mercury at 2 levels in blind duplicate samples of swordfish, tuna, oyster, and shrimp tissues. Both fortified and unfortified samples were analyzed. Methyl-bound mercury in the samples ranged from 0.15 to 2.48 μg Hg/g. The reproducibility coefficients of variation for the 8 samples ranged from 3 to 13%. The accuracy, measured by comparison to reference values, ranged from 99 to 120%. Reference values were determined in the Associate Referee's laboratory by replicate analyses of the fortified and unfortified samples. The method has been adopted official first action.

Mercury, a toxic element, is widely distributed in the environment. Human exposure to mercury occurs mainly from eating seafood (1). Microbial action in natural waters and bottom sediments converts various forms of mercury to methyl mercury, which then bioaccumulates in fish. The major portion of mercury found in fish muscle is methyl mercury, the element's most toxic form (2). Official methods used to support the Food and Drug Administration's (FDA) 1.0 μg Hg/g guideline for mercury in fish and shellfish determine total mercury by digesting the sample in boiling mineral acid and then analyzing the resulting solution by cold vapor atomic absorption spectroscopy or colorimetry (3). An official method that is specific for methyl-bound mercury in fish and shellfish is currently needed.

During the past 2 decades, various extraction schemes for the isolation of methyl mercury and gas-liquid chromatographic (GLC) conditions for the determinative step have been developed (4–12). Although many of these methods may be used for seafood analysis, none of them was considered rapid, simple, and accurate enough for routine determination of methyl mercury in fish and shellfish. Therefore, FDA scientists tried to develop a more practical GLC method. To improve speed and accuracy, Watts et al. (13) developed an acetone and benzene filtration step which removed organic interferences from homogenized tissue before extraction of methyl mercury. To ensure reliability of the Watts et al. GLC determinative step, a column conditioning procedure was developed by O'Reilly (14). To save time and eliminate possible losses during quantitative transfer, we substituted a shake, centrifuge, and decant procedure for the Watts et al. filtration step. To minimize chromatographic sample-column interaction, which produces unidentified late-eluting peaks for some seafood types, we began using only silanized supports with the O'Reilly column treatment procedure. Several suggestions from individuals who had used the Watts et al. method were also incorporated into the final analytical procedure. The method described here is the result of these efforts and is the procedure used in the collaborative study.

Collaborative Study

Nine collaborators, representing government, industry, and academic laboratories, agreed to participate.

Sample Selection

Two species of fish and 2 species of shellfish were selected for study. Swordfish was chosen because it frequently contains high levels of methyl mercury. Oyster was selected because it presents more problems during analysis for

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methyl mercury than other seafood (12). Tuna and shrimp were chosen to represent commonly consumed fish and shellfish.

Swordfish and tuna samples, both unfortified and fortified at one methyl mercury level, were analyzed. Oyster and shrimp were analyzed for methyl mercury at 2 fortification levels. The 8 levels were selected to demonstrate the suitability of the method for samples containing from 0.15 to 2.48 μg Hg/g. Each collaborator analyzed blind duplicates of all 8 levels, a total of 16 samples.

Sample Preparation

Canned shucked oyster and cooked-peeled-frozen shrimp samples were purchased locally. Whole, fresh-frozen tuna was obtained from a Los Angeles packing plant. Fresh-frozen swordfish was received from an FDA field inspector. The tuna and swordfish were thawed, skinned, and deboned in the Associate Referee's laboratory.

For each seafood type, ≥1.6 kg homogeneous sample was prepared. Tuna and shrimp were homogenized by grinding twice with a meat grinder followed by mixing in a Waring blender with water equal to about 25% the weight of the tissue. Results for tuna and shrimp are based on diluted samples. Swordfish and oysters (including packing liquid) were homogenized in a Waring blender with no water added. Results for swordfish and oyster are based on undiluted samples.

Each homogeneous sample was then divided into 2 equal portions. Each portion was either fortified with methyl mercuric chloride dissolved in isopropanol or treated only with isopropanol. After fortification and alcohol addition, both portions of each homogeneous sample contained the same amount of isopropanol. The 8 individual portions were thoroughly mixed in a Waring blender. Each portion was then divided into 3 g subsamples in 20 mL scintillation vials. All vials were labeled with a 6-digit code that identified the fish type and fortification level. The subsamples were frozen until time of analysis.

Collaborative Materials

In addition to the 16 samples, each collaborator received a copy of the method, report forms, AOAC special instructions for collaborators, a questionnaire, 0.4 g methyl mercuric chloride crystals for standard solution preparation, and 20 g practice sample. Each collaborator was instructed to obtain results within the range stated on the practice sample label before analyzing the collaborative samples.

Sample Homogeneity

Four to 6 subsamples from each of the 8 levels were analyzed in duplicate in this laboratory to verify subsample homogeneity and provide reference values. A one-way analysis of variance shows that there was no significant difference (95% confidence level) between individual subsamples of any level except shrimp level 1. This one statistically significant result is an artifact of the very small within-subsample variability (within-subsample coefficient of variation (CV) = 0.98%) found for shrimp level 1 and is not chemically significant. Shrimp level 1 subsamples were therefore considered adequately homogeneous for collaborative study.

Methyl Mercury in Fish and Shellfish
Gas-Liquid Chromatographic Method
Official First Action

25.D05 ***Principle***

Org. interferences are removed from homogenized sample by acetone wash followed by benzene wash. Protein-bound Me Hg is released by addn of HCl and extd into benzene. Benzene ext is concd and analyzed for CH_3HgCl by GLC.

25.D06 ***Reagents***

(**a**) *Solvents.*—Acetone, benzene, and isopropanol are all distd in glass (Burdick & Jackson Laboratories, Inc.; MCB Manufacturing Chemists, Inc.). *Note:* Benzene is a possible carcinogen.

(**b**) *Hydrochloric acid soln (1 + 1).*—Add concd HCl to equal vol. of distd or deionized H_2O and mix. Ext HCl soln 5 times with ¼ its vol. of benzene by shaking vigorously 15 s in separator. Discard benzene exts. Soln may be mixed in advance but must be extd immediately before use.

(**c**) *Carrier gas.*—GLC quality Ar-CH_4 (95 + 5).

(**d**) *Sodium sulfate.*—Heat overnight in 600° furnace, cool, and store in capped brown bottle. Line cap with Al foil to prevent contamination from cap.

(**e**) *Methyl mercuric chloride std solns.*—Keep tightly stoppered. (*1*) *Stock std soln.*—1000 μg Hg/mL. Weigh 0.1252 g CH_3HgCl (ICN-K&K Laboratories, Inc., Plainview, NY 11803) into 100

mL vol. flask. Dil. to vol. with benzene. (2) *High intermediate std soln.*—40 μg Hg/mL. Dil. 10.0 mL stock soln to 250.0 mL with benzene. (3) *Low intermediate std soln.*—2.0 μg Hg/mL. Dil. 10.0 mL high intermediate std soln to 200.0 mL with benzene. (4) *Working std solns.*—0.010–0.30 μg Hg/mL. Prep. monthly by dilg with benzene in vol. flasks as follows: Dil. 15 mL of 2.0 μg Hg/mL std to 100.0 mL, 10.0 mL to 100.0 mL, and 10.0 mL to 200.0 mL for 0.30, 0.20, and 0.10 μg Hg/mL, resp. Dil. 20 mL of 0.10 μg Hg/mL std to 25.0 mL, 10.0 mL to 25.0 mL, 10.0 mL to 50.0 mL, and 10.0 mL to 100.0 mL for 0.080, 0.040, 0.020, and 0.010 μg Hg/mL, resp.

(**f**) *Mercuric chloride column treatment soln.*—1000 ppm $HgCl_2$. Dissolve 0.1 g $HgCl_2$ in 100 mL benzene.

25.D07 *Apparatus*

Wash all glassware with detergent (Micro Laboratory Cleaner, International Products, Trenton, NJ 08601, or equiv.) and rinse thoroly with hot tap H_2O followed by distd or deionized H_2O.

(**a**) *Centrifuge.*—Model UV (International Equipment Co., Needham Heights, MA 02194), or equiv.

(**b**) *Centrifuge tubes.*—50 mL capacity with ground glass or Teflon-lined stoppers.

(**c**) *Kuderna-Danish (K-D) concentrators.*—250 mL flask (No. K570001, Kontes Glass Co.) and 10 mL graduated concentrator tube (No. K570050, size 1025, Kontes Glass Co.).

(**d**) *Modified Snyder distilling column.*—Modify Snyder column (No. K503000 size 121, Kontes Glass Co.) in either of 2 ways: (*i*) Shorten 3-section, 3-ball column to 2-section, 2-ball column by cutting off top at uppermost constriction. (*ii*) Insulate 3-section, 3-ball column by wrapping glass wool around top section and holding it in place with Al foil. Glass wool and foil must surround only top section above top ball.

(**e**) *Carborundum boiling chips.*—20 mesh, HCl-washed.

(**f**) *Graduated cylinders.*—Class A, 25 mL capacity, with ground-glass stopper (Kimble 20036, or equiv.).

(**g**) *Transfer pipets.*—Disposable glass, Pasteur-type 5¾ in. long (No. 13-678-6A, Fisher Scientific Co., or equiv.).

(**h**) *Dropping pipets.*—5 mL capacity (No. 13-710B, Fisher Scientific Co., or equiv.).

(**i**) *Gas chromatograph.*—Hewlett-Packard Model 5710A or equiv., equipped with linear ^{63}Ni electron capture detector and 6 ft × 2 mm id silanized glass column packed with 5% DEGS-PS on 100–120 mesh Supelcoport (Supelco, Inc.). Pack column *no closer* than 2.0 cm from injection and detector port nuts and hold packing in place with 2 cm high quality, silanized glass wool at both ends. Install oxygen scrubber and molecular sieve dryer (No. HGC-145, Analabs, North Haven, CT 06473, or equiv.) between carrier gas supply and column. Condition column according to manufacturer's instructions as follows: Flush column 0.5 h with carrier gas flowing at 30 mL/min at room temp. Then heat 1 h at 100°. Next, heat column to 200° at programmed heating rate of 4°/min and hold at 200° overnight. Do not connect column to detector during this conditioning process. Maintain 30 mL/min carrier gas flow at all times during conditioning, treatment, and use. Operating conditions: column 155°; injector 200°; detector, 300°; carrier gas flow 30 mL/min; and recorder chart speed 0.5–1.0 cm/min. Under these conditions and with $HgCl_2$ column treatment procedure described below, CH_3HgCl peak will appear 2–3 min after sample injection.

25.D08 *Mercuric Chloride Column Treatment*

5% DEGS-PS conditioned according to manufacturer's instructions can be used to det. CH_3HgCl only after treatment by $HgCl_2$ soln, (**f**). Treat column any time column has been heated to 200°. Because column performance degrades with time, also treat column periodically during use. Perform appropriate $HgCl_2$ treatment procedures described below. Procedure (**b**) produces most stable baseline and is recommended over procedure (**c**) for routine use.

(**a**) *Following 200° column conditioning.*—If column has just been conditioned overnight at 200°, use this procedure. Adjust column temp. to 160° and connect detector. When baseline is steady, treat column by injecting 20 μL $HgCl_2$ treatment soln 5 times at 5–10 min intervals. (Change in column performance may be monitored by injecting 5 μL 0.010 μg Hg/mL std soln before and between $HgCl_2$ treatment soln injections.) During treatment procedure, large broad peaks will elute. (CH_3HgCl peak retention time will decrease and peak ht will increase.) Approximately 1½–1¾ h after last $HgCl_2$ treatment soln injection, a final large peak will elute. (CH_3HgCl peak ht and retention time will be stable.) This broad peak and CH_3HgCl peak ht stability signal completion of treatment process. Adjust column temp. to 155° and wait for steady baseline; then column is ready for use.

(b) *On day preceding sample extract analysis.*—If column has been treated by procedure (a) or used at 155° to analyze sample exts, column may be treated at end of working day for next day's use as follows: Lower column temp. to 115° and inject 20 μL $HgCl_2$ treatment soln *one* time. Broad peaks will elute between 11 and 15 h after $HgCl_2$ injection. Next working day, increase column temp. to operating temp. When baseline is steady (ca 15–30 min), column is ready for use.

(c) *During sample extract analysis at 155°.*—If column has been used at 155° for ext analysis and column performance has degraded enough to require $HgCl_2$ treatment, increase column temp. to 160°, inject *one* 20 μL aliquot of $HgCl_2$ treatment soln, and monitor baseline. Large, broad peaks will elute 1–1½ h after $HgCl_2$ injection, signaling completion of treatment process. Decrease column temp. to 155° and wait for steady baseline; then column is ready for use.

25.D09 *Extraction of Methyl Mercury Chloride*

Perform all operations, except weighing, in laboratory hood. Accurately weigh 2 g homogenized sample into 50 mL centrf. tube. Add 25 mL acetone, stopper, and shake vigorously 15 s. Remove stopper, cover with foil, and centrf. 2–5 min at 2000 rpm. Carefully decant and discard acetone. (Use dropping pipet to remove acetone, if necessary.) Repeat 25 mL acetone wash step twice more. Break up tissue with glass stirring rod before shaking, if necessary. Add 20 mL benzene, stopper, and shake vigorously 30 s. Remove stopper, cover with foil, and centrf. 2–5 min at 2000 rpm. Carefully decant (or draw off with dropping pipet) and discard benzene. Extraneous peaks in final GLC anal. chromatograms indicate that more vigorous shaking with acetone and benzene is required.

Add 10 mL HCl soln to centrf. tube contg acetone and benzene-washed sample. Break up tissue with glass stirring rod, and ext sample by adding 20 mL benzene and shaking gently but thoroly 2 min. Remove stopper, cover with foil, and centrf. 5 min at 2000 rpm. If emulsion forms, add 2 mL isopropanol and gently stir benzene layer to break emulsion, taking care not to disturb aq. phase, and recentrf. Carefully transfer benzene layer to K-D concentrator, using 5 mL dropping pipet. Rinse centrf. tube walls with 3–4 mL benzene and transfer rinse to K-D concentrator. Repeat extn step twice more, adding 20 mL benzene and shaking 1 min each time. Combine all 3 benzene exts in K-D concentrator.

Place 4–6 boiling chips in K-D concentrator, connect Snyder column, wet Snyder column bubble chambers with 3–4 drops of benzene, and immediately place tube in steam bath or vigorously boiling H_2O bath. Evap. so that 8 mL remains when cooled to room temp. Cool. Disconnect concentrator tube and quant. transfer soln to 25 mL g-s graduate using Pasteur-type transfer pipet. Dil. to 20.0 mL with benzene and mix. Add 4 g Na_2SO_4 and mix again. Na_2SO_4 must be added to 20 mL concd sample ext within 10 h of first acetone wash. Tightly stoppered exts may be held overnight at this point. Analyze by GLC.

25.D10 *Gas-Liquid Chromatography*

Verify that system is operating properly by injecting 5 μL vols. of 0.01 μg Hg/mL working std soln into chromatograph. Difference between CH_3HgCl peak hts for 2 injections should be ≤4%. Check detector linearity by chromatographing all 0.01–0.30 μg Hg/mL working std solns.

Inject duplicate 5 μL vols. (equiv. to 0.5 mg sample) of ext. Difference between CH_3HgCl peak hts for 2 injections should be ≤4%. Next, inject duplicate 5 μL vols. of std soln with CH_3HgCl concn approx. equal to or slightly greater than ext CH_3HgCl concn. Because column performance and peak ht slowly decrease with time, calc. each sample concn by comparison to std soln injected immediately after sample.

Calc. Me Hg content of homogenate in μg Hg/g (ppm Hg) by comparing av. CH_3HgCl peak ht of duplicate sample injections with av. CH_3HgCl peak ht of duplicate std injections.

$$\text{ppm Hg} = (R/R') \times (C'/C) \times 20$$

where R = av. peak ht of duplicate sample injections; R' = av. peak ht of duplicate std injections; C = g sample; C' = concn of Hg in CH_3HgCl std soln (μg Hg/mL).

Results

All 9 collaborators completed the study. All collaborators provided chromatograms except Collaborator 4. The results used for statistical evaluation are presented in Table 1.

Collaborator 8 was excluded as a participant because the analyst did not follow the method as directed. This collaborator made the following

Table 1. Collaborative results for the determination of methyl mercury (μg Hg/g) in fish and shellfish by GLC

	Swordfish		Tuna		Oyster		Shrimp	
Coll.	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
Reference[a]	1.351	2.482	0.145	0.876	0.255	0.574	0.400	1.008
1	1.37	2.65	0.15	0.95	0.33[b]	0.84	0.45	1.23
	1.43	1.69	0.17	0.95	0.97[b]	0.76	0.42	1.19
2	1.39	2.62	0.13	1.10	0.29	0.62	0.47	1.02
	1.40	2.62	0.15	0.98	0.33	0.61	0.42	1.26
3	1.42	2.62	0.14	0.80	0.26	0.56	0.49	1.25
	1.32	2.61	0.15	1.01	0.26	0.62	0.48	0.99
4	1.30	2.55	0.44[c]	0.71	0.58[c]	1.17[c]	0.51	1.14
	1.34	2.47	0.26[c]	0.71	0.53[c]	0.92[c]	0.39	1.11
5	1.32	2.76	0.14	1.06	0.29	0.70	0.41	1.13
	1.38	2.63	0.15	0.94	0.29	0.69	0.44	1.10
6[d]	0.98	4.20	0.40	2.83	0.34	0.33	0.84	2.41
	2.33	1.87	0.42	2.09	0.64	0.42	0.85	2.40
7	1.32	2.00	0.16	0.84	0.33	0.79	0.42	1.02
	1.40	2.21	0.15	0.89	0.28	0.72	0.42	1.03
9	1.42	2.43	0.12	1.00	0.24	0.75	0.50	1.02
	1.41	2.47	0.17	1.00	0.29	0.63	0.42	1.12
Coll. av.	1.373	2.452	0.148	0.924	0.290	0.691	0.446	1.115
Accuracy, %[e]	102	99	102	105	114	120	112	111
Repeatability								
S_o	0.043	0.226	0.017	0.073	0.026	0.050	0.042	0.100
CV_o, %	3	11	12	8	9	7	10	9
Coll. av.								
S_L	0.013	0.132	0	0.099	0.017	0.072	0	0
CV_L, %	1	5	0	11	6	10	0	0
Reproducibility								
S_x	0.045	0.297	0.017	0.123	0.031	0.087	0.042	0.100
CV_x, %	3	12	12	13	11	13	10	9

[a] Average of results from Associate Referee's laboratory, n = 8–12.
[b] Data determined to be outliers by Cochran test; not used in statistical evaluation.
[c] Data determined to be outliers by Dixon test on blind duplicate average values; not used in statistical evaluation.
[d] Data determined to be outliers by Youden rank-sum test; not used in statistical evaluation.
[e] Calculated by comparison of collaborator average value with reference value.

modifications in the method: (a) used <2 g homogenate for determinations, (b) performed replicate determinations on each subsample, and (c) evaporated (did not decant) acetone and benzene before isolation of methyl mercury.

Questionnaire Response

All collaborators used 5% DEGS-PS on 100–120 mesh Supelcoport. The column used by Collaborator 9 was packed by the manufacturer. All other columns were packed in the collaborators' laboratories with stock packing (liquid phase loaded onto solid support by the manufacturer). Four different stock packing lot numbers were used by the collaborators.

Seven different chromatograph models produced by 4 manufacturers were used for analysis. Collaborator 9 reported that detector response deviated from linearity for standard solutions >0.10 μg Hg/mL and that it was necessary to analyze diluted swordfish extracts (final volume equivalent to 40 and 60 mL for levels 1 and 2, respectively) to obtain results within the linear range of standard solution response. This problem was not reported by any other collaborator nor experienced in this laboratory. Chromatograms were recorded on 6 different recorder models produced by 4 manufacturers.

Collaborator 4 had <6 months' experience with GLC. Collaborator 1 had 6 months to 1 year of experience. All other collaborators had worked with GLC 2 years or more.

Collaborators kept the extracts different lengths of time between the addition of sodium sulfate and GLC analysis. Collaborator 6 kept some extracts more than 7 days because of instrument failure. All extracts were held 2–3 days by Collaborator 4. Collaborator 9 kept some extracts 2–3 days and the remainder for 1 day. All other collaborators held extracts 1 day or less.

Rejection of Outliers

The results from Collaborator 6 were excluded from statistical evaluation because (a) some standard solution peak heights were not pro-

portional to concentration, indicating possible error in serial dilution; (b) for the same standard solution, average peak heights of duplicate injections varied greatly during short periods of time, indicating possible instrument malfunction; and (c) complete instrument breakdown occurred during analysis. The Youden rank-sum test (15) was applied to the data to determine whether the problems encountered by Collaborator 6 were great enough to cause pronounced systematic error. This test assigns rank numbers to the 8 blind duplicate average values for each level (lowest value = rank 1, second lowest value = rank 2, etc.) and then sums the rank numbers for each collaborator. The statistical distribution of rank-sums for ideal data when there is no systematic error has upper and lower limits. A collaborator rank-sum higher than the upper limit indicates that the collaborator has a greater number of high results than can be expected by chance alone. Conversely, a collaborator rank-sum lower than the lower limit indicates a greater number of low results than can be expected by chance. Application of the Youden rank-sum test (95% confidence level) to the results in Table 1 indicates that data from Collaborator 6 are indeed outliers due to systematic error, consistently yielding high results. Exclusion of results from Collaborator 6 from statistical evaluations was therefore justified.

The Dixon test (15) was applied to results from the remaining 7 collaborators to identify outlying blind duplicate average values. In the Dixon test, the gap between the suspected outlier and the nearest value is expressed as a fraction of the range of values. For an ideal set of data in which there are no outlying results, the fraction does not exceed a value specified by the number of results in the set and the confidence level chosen. If the fraction calculated from a collaborator's data exceeds the Dixon value, the collaborator's data may be rejected. Application of the Dixon outlier test (95% confidence level) to the 7 blind duplicate average values for each level indicates that tuna level 1 and oyster level 2 results from Collaborator 4 are outliers (values too high). These results were therefore eliminated from further statistical evaluation.

The results for oyster level 1 required closer inspection. Although the blind duplicate average value from Collaborator 1 is very high, it is not rejected by Dixon test application to the 7 collaborator averages calculated from 14 blind duplicate results. High results from Collaborators 1 and 4 instead mask each other from Dixon test rejection. The Cochran test (15) was therefore applied to oyster level 1 results to look for individual collaborators whose readings have too large a variability (i.e., spread or variance). In this test, the variance is calculated for the blind duplicates from each collaborator. The largest variance is then expressed as a fraction of the sum of the variances. If the fraction calculated from a collaborator's data exceeds the Cochran value (specified by the number of collaborators in the set and the confidence level chosen) the collaborator's data may be rejected. Application of the Cochran test (95% confidence level) to the 14 blind duplicate oyster level 1 results indicates that the data from Collaborator 1 are indeed Cochran outliers and should be eliminated.

The Dixon test was then applied to the remaining 6 collaborator averages calculated from 12 blind duplicate results. When oyster level 1 data are treated in this way, the data from Collaborator 4 are once again outliers and the outlying values are once again high. The fact that all 3 blind duplicate average Dixon outliers are from Collaborator 4 and that these outlying values are all high suggests that Collaborator 4 may be an outlying analyst and that this special treatment of oyster level 1 data is justified. The values of Collaborators 1 and 4 for oyster level 1 were therefore eliminated from further statistical evaluation.

Statistical Evaluation

The data in Table 1, purged of outlying data (namely, from Collaborator 6 for all samples, Collaborators 1 and 4 for oyster level 1, and Collaborator 4 for tuna level 1 and oyster level 2), were statistically evaluated. Within- and between-laboratory variabilities were estimated by a one-way analysis of variance. The results are presented in Table 1. Repeatability standard deviation, S_o; pure between-laboratory standard deviation, S_L; reproducibility standard deviation, S_x; and the corresponding CV values are defined by Steiner (15) and have been described previously by workers in this laboratory (16). The CV_x range for the 8 levels analyzed in this study, 3–13%, is very good. Historically, CV_x values at ppm levels have been about 16% for AOAC studies (17).

Reference values established by replicate analyses in this laboratory were used to evaluate the accuracy of the collaborative results. Accuracy was calculated as percent by dividing the average of collaborative values by the reference value. These results are also shown in Table 1. The range of accuracy is 99–120%. The overall average accuracy, 108%, is slightly high.

Discussion

The quantitation limit of the GLC method depends on the adequacy of sample cleanup. For oyster tissue, the worst-case sample analyzed in this study, the quantitation limit is 0.05 μg Hg/g. At lower levels, extraneous background peaks begin to dominate chromatograms so that identification and measurement of the methyl mercuric chloride peak are questionable. At the 0.05 μg Hg/g level, the methyl mercuric chloride peak is 10 times larger than extraneous background peaks in oyster tissue. This limit is based on a 5 μL injection of 2 g sample in 20 mL benzene extract.

The positive bias in collaborator accuracy may be due to incomplete removal of organic interferences by the acetone/benzene wash step and to holding extracts which contain these interferences too long before analysis by GLC. Visual inspection shows that, in general, chromatograms obtained by collaborators contained more extraneous peaks than those produced in this laboratory. Extraneous peaks in chromatograms submitted by the collaborators appear at retention times similar to extraneous peaks produced during ruggedness testing of the acetone/benzene wash step. Our ruggedness test on fortified and unfortified homogenates showed that less vigorous shaking with acetone and benzene before methyl mercury extraction produces extraneous peaks in tuna, shrimp, and oyster chromatograms. If the acetone and benzene wash is eliminated altogether, the methyl mercury and extraneous peaks on a chromatogram from an oyster sample are indistinguishable. When an extraneous peak elutes at the same time as the methyl mercury peak, a positive error in peak height measurement is made. Extraneous peaks do not appear in either acetone/benzene washed or unwashed swordfish chromatograms.

The ruggedness test also showed that oyster, tuna, shrimp, and swordfish extracts that produce chromatograms without extraneous peaks can be kept for 7, 14, 14, and >14 days, respectively, with no change in methyl mercury concentration. Oyster extracts that produce many extraneous peaks can be held for only 3 days before the apparent methyl mercury concentration increases. The apparent concentration increase in aged extracts is probably due to formation of unidentified interfering compounds that elute at the same time as methyl mercury. Because some collaborators' chromatograms and results may have been improved by more vigorous shaking with acetone and benzene before methyl mercury extraction, this instruction has been added to the official method.

Recommendation

It is recommended that the electron capture gas-liquid chromatographic method for the determination of methyl mercury in fish and shellfish be adopted official first action.

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Electrothermal Atomic Absorption Spectrometric Determination of Selenium in Foods and Diets

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The validity of 2 electrothermal atomic absorption spectrometric methods for determination of selenium in foods and diets was tested. By using 0.5% Ni(II) as a matrix modifier to prevent selenium losses during the ashing step, it was shown that selenium can be determined in samples containing ≥1 μg Se/g dry wt without organic extraction. The mean recovery tested, using NBS Bovine Liver, was 98%; recovery of added inorganic selenium in Bovine Liver matrix was 100%. In addition, this method gave values closest to the median value of all participating laboratories using hydride generation AAS or the spectrofluorometric method in a collaborative study on high selenium wheat, flour, and toast samples. For samples with concentrations <1 μg Se/g dry wt, separation of selenium from interfering Fe and P ions by organic extraction was necessary. Using inorganic ^{75}Se in meat and human milk matrixes, an ammonium pyrrolidine dithiocarbamate-methyl isobutyl ketone-extraction system with added Cu(II) as a matrix modifier yielded the best extraction recoveries, 97 and 98%, respectively. Accuracy and precision of the method were tested using several official and unofficial biological standard materials. The mean accuracy was within 4% of the certified or best values of the standard materials and the day-to-day variation was 9%. The Se/Fe or Se/P interference limits proved to be low enough not to affect selenium determinations in practically all foods or diets. The practical detection limit of the method was 3 ng Se/g dry wt for 1.0 g dry wt samples. Approximately 20 duplicate determinations can be performed per day. In terms of sensitivity, accuracy, precision, and sample throughput, the tested method compares favorably with the best methods available.

Growing interest in selenium as an essential trace element for humans has given importance to assessing the selenium contents of various foods and diets to estimate the dietary selenium intakes among different populations. Numerous analytical techniques can be employed for the determination of selenium in biological materials; the most frequently used are spectrofluorometry, hydride generation atomic absorption spectrometry (AAS), and neutron activation analysis (NAA). In general, however, the spectrofluorometric method is cumbersome and the AAS method using hydride generation has been reported to suffer from matrix interferences when applied to such complex matrixes as foods and diets (1–5). Further evidence on matrix interference problems is provided by interlaboratory comparison studies on selenium levels in various biological materials (5–7). These studies have demonstrated problems in both accuracy (5, 6) and precision (5–7). Therefore, a method applicable to routine determinations not requiring costly or special instrumentation and free of matrix interferences is needed.

Using nickel as a matrix modifier to prevent selenium losses during the ashing step, an electrothermal atomic absorption spectrometric (ETAAS) method fulfilling these criteria has recently been developed for serum or plasma selenium (8). After preliminary digestion, this method can also be used to determine selenium in foods sufficiently high in selenium to allow extensive dilution, e.g., selenium nutritional supplements at the mg/kg concentration level (9).

However, because of spectral interferences at the 196 nm selenium absorption line caused by iron and phosphorus, this method cannot generally be applied to foods and diets low in selenium and high in these matrix elements (10–12). Several procedures have been proposed to separate selenium from these interfering elements. They include methods specifying cation exchange (13), precipitation of selenium with ascorbic acid (14), and extraction of chelated selenium into an organic solvent (15–17).

Recently, a method was presented in which the interfering cations are eliminated by chelating with ethylenedinitrotetraacetate (EDTA) followed by separation of selenium by an ammonium pyrrolidine dithiocarbamate-methyl-isobutyl ketone (APDC-MIBK) extraction system (18). This procedure offers certain advantages over the other published extraction methods (15–17) because the copper(II) ions used for ma-

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trix modification to increase the temperature during the ashing step for improved sensitivity can be co-extracted with selenium. Although the validity of this procedure was preliminarily tested for some foods, we felt that a more comprehensive systematic study was required. The present paper describes the results of such a study using different types of biological standard reference materials (SRM) for verification.

In addition, the applicability of ETAAS for determination of selenium, without organic extraction, using nickel(II) ions for matrix modification was tested for the determination of selenium in nutritional supplements by a certified biological standard material and by an interlaboratory collaboration.

METHODS

Samples

The validity of the method using the APDC-MIBK-extraction system was tested by using certified international standard materials—National Bureau of Standard's (NBS) Bovine Liver (Code SRM 1577) and International Atomic Energy Agency's (IAEA) animal muscle (Code H-4). In addition, the following unofficial Finnish biological standard materials previously used as intercalibration samples were analyzed for selenium: wheat flour (Code 2); dried ground beef (Code 5); dried low fat milk (Code 6) and dried human milk (Code 3). All these standard samples were the same materials and codes as described in a recent intercalibration study by Varo and Koivistoinen (5). In addition, a frozen sample of pooled serum previously used as an intercalibration material (6) was analyzed for selenium.

The applicability of the method without extraction for high selenium nutritional supplements was tested using NBS Bovine Liver, samples of flour, and a toast (made of wheat grown in a high selenium soil in South Dakota) and with a selenium-rich yeast. In addition to our laboratory, the yeast and cereal samples were distributed for laboratory intercomparison to 4 Finnish laboratories regularly performing selenium determinations. One of these laboratories used fluorometric assay while the rest used hydride generation AAS.

Apparatus

(a) *Atomic absorption spectrophotometer.*—Model 5000 spectrometer equipped with deuterium arc lamp, Model PRS-10 printer, and Model 56 recorder (all Perkin-Elmer).

(b) *Graphite furnace.*—Model HGA-500 graphite furnace equipped with Model As-40 Autosampler (Perkin-Elmer).

(c) *Selenium lamp.*—Perkin-Elmer selenium electrodeless discharge lamp equipped with Perkin-Elmer power supply.

(d) *Gamma counter.*—Model Ultragamma 1280 automatic gamma counter (LKB-Wallac) coupled with a teletype.

Reagents

(a) *Water.*—Use double distilled water or comparable stored in high pressure polyethylene container for preparation of reagents and standard solutions.

(b) *Nickel nitrate solution.*—Weigh 251.45 g $Ni(NO_3)_2 \cdot 6H_2O$ into 1 L volumetric flask and dilute to volume with water to yield 5% (w/v) Ni(II) solution.

(c) *Selenium standard solutions.*—

(1) *Stock solution.*—1.000 g Se/L. Weigh 1.405 g SeO_2 and dissolve in 1 mL HCl. Transfer solution quantitatively into 1 L volumetric flask, dilute to volume with water, and mix thoroughly.

(2) *Intermediate solution.*—1 mg Se/L. Using automatic pipet with disposable polyethylene tips, pipet 100 μL stock solution into 100 mL volumetric flask and dilute to volume with water.

(3) *Working standards for extraction method.*—Pipet 1 mL 1 μg Se/mL standard solution into 100 mL volumetric flask and dilute to volume with water to yield 10 ng Se/mL solution.

(4) *Working standards for method without extraction.*—Pipet 1, 2, and 4 mL 1 μg Se/mL standard solution into 100 mL volumetric flasks, followed by 20 mL 5% Ni(II) solution, and dilute to volume with water to yield 10, 20, and 40 ng Se/mL in 1.0% Ni(II) solution.

(d) *Copper sulfate solution.*—Weigh 0.390 g $CuSO_4 \cdot 5H_2O$ into 100 mL volumetric flask and dilute to volume with water to 1 mg Cu/mL solution.

(e) *Ethylenedinitrotetraacetate solution.*—Weigh 5 g EDTA into 100 mL volumetric flask and dilute to volume with water.

(f) *Methyl isobutyl ketone.*—Saturate with water before use.

(g) *Ammonium pyrrolidine dithiocarbamate solution.*—Weigh 2 g APDC into 100 mL volumetric flask and add water. Dilute to volume when solubilized and transfer to 250 mL separatory funnel, add 10 mL MIBK solution and shake 2 min. Let phases separate and collect water phase.

Table 1. Instrumental parameters

Model 5000 spectrometer:						
Wavelength					196.1 nm	
Slit					0.7 nm (low)	
D_2-background correction					yes	
Mode					peak height	
Integration time					3 s	
Scale expansion					10	
Printout					yes	
HGA-500 programs:						
	Temperature (°C)		Ramp (s)		Hold (s)	
Step	Prog. 1	Prog. 2	Prog. 1	Prog. 2	Prog. 1	Prog. 2
1 (dry)	110	120	5	10	10	30
2 (ash)	1000	1200	10	30	30	30
3 (atomize)	2500	2700	0	0	3	3
Step	Read	Baseline	Internal flow			
1 (dry)			300			
2 (ash)			300			
3 (atomize)	0	−5	10			
Other analytical conditions:						
Type of graphite tube					normal (Prog. 1) pyrolytic (Prog. 2)	
Purge gas					argon	
Optical temperature sensor (graphite furnace)					yes	
Sample injection volume					20 μL	

(**h**) *Ammonium dibasic citrate buffer solution.*—Prepare 0.1M solution (pH 5) by weighing 22.6 g ammonium dibasic citrate (Merck) into 1 L water.

(**i**) *Bromophenol blue indicator.*—Weigh 40 mg bromophenol blue into 100 mL flask and dilute to volume with ethanol.

(**j**) *Selenium radioisotope.*—$Na_2{}^{75}SeO_3$ in water (0.6 mCi/mL, Amersham, Code SCS1).

Preparation of Standards and Samples for Instrumental Analysis

To determine selenium using MIBK-extraction, prepare working standards by adding 1, 2, 5, and 10 mL of 10 ng Se/mL standard solution into 100 mL stopcock test tubes. Add 10 mL water and perform extraction procedure as described for samples to obtain 2, 4, 10, and 20 ng Se/mL calibration standards. Use 2 blanks treated like the samples for each set of determinations.

Use method of standard additions to determine selenium in samples whose concentration is ≥1 mg Se/kg dry wt. Pipet 0.5 mL aliquots of each sample into 4 sample cups containing 0.5 mL Se standards of 0, 10, 20, and 40 μg Se/mL in 1% Ni(II) solutions to yield additions of 0, 5, 10, and 20 μg Se/mL in 0.5% Ni(II) solution.

Instrumental Analysis

The instrumental parameters for both selenium in MIBK matrix (furnace program 1) and water matrix (furnace program 2) are presented in Table 1.

Calculation

When using method of standard additions, calculate concentration of unknown sample as follows: Using pocket calculator or computer, define standard additions curve by calculating linear regression from addition-absorbance data.

When using extraction method, determine concentration of original sample as follows: On pocket calculator, use least squares method to set up calibration curve with external standards and define concentration of sample solutions from calibration curve.

Digestion Methods

For digestion of samples to be analyzed for selenium by the extraction method, the following procedure was used: Depending on selenium concentration, weigh 0.2–1.0 g dry weight into wet digestion tube, followed by 10 mL digestion acid mixture containing 6 mL 65% HNO_3, 2 mL 70% $HClO_4$, and 2 mL 95% H_2SO_4.

Table 2. Results of collaborative study on Se levels (mg/kg) in nutritional supplements

Laboratory code[a]	Sample		
	Flour	Toast	Se-enriched yeast
A	5.8	4.7 ± 0.2 (n = 3)	169 ± 24 (n = 4)
B	4.2	3.0 ± 0.1 (n = 4)	103 ± 2[b] (n = 2)
C	5.2	3.1 ± 0.05 (n = 4)	113 ± 3[b] (n = 2)
D	5.2	3.7 ± 0.3 (n = 4)	129 ± 3[b] (n = 2)
This method	4.6	3.6 ± 0.2 (n = 14)	127 ± 2.8 (n = 10)
Mean ± SD	4.8 ± 0.7	3.6 ± 0.7	128 ± 25

[a] Laboratories A, B, and C used hydride generation AAS; Laboratory D used spectrofluorometry and ^{75}Se to check the recovery after a perchloric acid digestion.
[b] Standard error of the mean.

Heat mixture carefully in wet digestion apparatus almost to boiling, and keep at this temperature overnight or at least 8 h. Evaporate remaining HNO_3 and part of the $HClO_4$ by raising temperature to the boiling point until 2–3 mL solution remains. After cooling, complete digestion by adding 5 mL 30% H_2O_2 to solution and heating it to boiling point. Add 10 mL 10M HCl and boil 17 min at 100°C to reduce any selenium(VI) to selenium(IV).

For digestion of selenium nutritional supplements, use of $HClO_4$ or H_2SO_4 is not necessary. Boil samples (0.2–1 g dry wt) overnight in 20 mL 65% HNO_3, cool, and dilute with water to give approximately twice the concentration (10–15 ng Se/mL) optimal for instrumental measurement.

Extraction Procedure

Use 0.04% bromphenol blue as an indicator, and adjust pH of solution to 4–5 by adding NH_4OH. Add 5 mL 0.1M ammonium dibasic citrate buffer solution (pH 5) followed by 5 mL 5% EDTA solution. Add 100 μL 1.0 mg/mL copper(II) solution and transfer entire solution into 100 mL stopcock test tube containing 2 mL 2% APDC solution. Finally, add 5 mL MIBK solution and shake mixture mechanically 5 min. Let organic phase separate 20 min before sampling.

Radioactivity Measurements

The 280 keV γ-peak of ^{75}Se was measured along with a standard. The counting time and ^{75}Se spiking were adjusted to allow a counting of at least 10 000 pulses. The background activity value used in the calculations was based on a 1 h counting time.

Results and Discussion

ETAAS Without Organic Extraction

The intercalibration study on selenium in nutritional supplements (Table 2) reveals an unexpectedly high variation among the participating laboratories, considering the high concentration level. Furthermore, the difference in reported values by participating laboratories is clearly due to systematic errors because corresponding differences exist in all samples tested.

Table 3. Accuracy and recovery of ETAAS method without organic extraction, tested using NBS Bovine Liver (SRM 1577)[a]

Sample No.	Se found, μg/g dry wt	Recovery, %	Recovery after 1 μg/g inorganic Se added	
			μg Se/g	Rec., %
1	1.055	95.9	2.137	101.8
2	1.201	109.2	2.116	100.8
3	0.939	85.4	2.034	96.9
4	1.223	111.1	2.125	101.2
5	0.985	89.6	2.100	100.0
Mean ± SD	1.081 ± 0.13	98.2 ± 11.5	2.125 ± 0.07	100.1 ± 1.9

[a] NBS certified value 1.1 ± 0.1 μg Se/g dry wt.

Table 4. Recovery after various extraction procedures of ^{75}Se added to meat or human milk samples [a]

Procedure	Recovery, %	
	Human milk	Meat
Digestion	97.0	96.8
EDTA + Cu(II) APDC–MIBK	98.4	97.2
Cu(II) + APDC-MIBK	93.1	95.9
EDTA + Ni(II) APDC-MIBK	82.1	—
EDTA + Cu(II) NaDDC-MIBK	95.1	—

[a] All experiments were performed in duplicate.

In particular, the results presented by laboratory A are clearly outlying compared with the other values, suggesting the vulnerability of the hydride generation AAS method to systematic errors. The results of the present ETAAS method are closest to the mean value of reported results for both types of sample. Moreover, by the present method, correct results were obtained for the NBS Bovine Liver with a mean value of 1.08 ± 0.13 mg/kg and a recovery of 98.2 ± 11.5% (Table 3). It may thus be concluded that the present method is free of interferences for the samples tested. The very high iron content of the NBS Bovine Liver matrix (270 mg/kg) did not result in erroneously low selenium analytical values for this material, suggesting that the selenium concentration level of 1 mg/kg dry wt is high enough to allow the determination of selenium in practically all foods whose selenium content is higher than that. This is further supported by a study in which, using a comparable method, good recovery was obtained for selenium in nutritional supplements after only a very short preliminary digestion (9).

Applicability of the APDC-MIBK-ETAAS Method

Recovery Studies.—To optimize the analytical procedure, recovery tests were carried out with inorganic radioactive ^{75}Se added to the digestion tubes together with meat (0.5 g dry wt) or human milk (1.0 mL) samples. The analytical procedure was performed either (*1*) as described above; or (*2*) using Ni(II) instead of Cu(II); or (*3*) without EDTA, or (*4*) using sodium diethyldithiocarbamate as the chelating agent. The recoveries were calculated based on γ-activity measurements as described in the methods section.

As Table 4 indicates, recovery was satisfactory for both the digestion (96.9%) and the proposed extraction method (98.4% for human milk and 97.2% for meat). However, when EDTA was omitted, the extraction recovery was somewhat lower for both meat (95.9%) and human milk (93.1%), indicating that EDTA clearly enhances the chelation of selenium with APDC by blocking the competition from cations in the system. Moreover, when Cu(II) was replaced with the same amount of Ni(II), the selenium extraction recovery was markedly lowered (82.1%). Although the reason for this is unknown, the advantage of Cu(II) over Ni(II) as a stabilizer is further supported by these results in addition to the fact that the Cu(II)-APDC complex is more stable than the Cu(II)-EDTA complex (18).

Finally, APDC seems to be a somewhat better chelating agent (98.4%) than NaDDC (95.1%) when tested in a digested human milk matrix (Table 4).

Accuracy and Precision.—Recently, Neve et al. (15) have shown that wet digestion with concentrated HNO_3 is not effective enough to decompose all selenium compounds naturally present in biological materials such as milk and liver. The present method requires that all the selenium in a sample be reduced to Se(IV) before chelation, so recovery tests using added inorganic ^{75}Se do not necessarily validate the digestion method used. Therefore, accuracy tests using various types of biological standards are necessary. In addition, possible spectral interferences in the instrumental analysis would thus be revealed in unacceptably low values.

Table 5 summarizes the results of accuracy and precision experiments, indicating both satisfactory accuracy and day-to-day variation for all reference materials tested. The mean values obtained for these standard samples were only 4% lower on average compared with the reference values, respectively. The day-to-day variation for all samples tested was 9.1% on average.

The present method was applied to the determination of selenium in 80 self-selected Finnish diets (Kumpulainen et al., manuscript in preparation). The mean and median selenium contents of these diets were 98 and 82 ng/g dry wt with a range of 47–300 ng/g. These concentrations represent mean and median selenium intakes of 57 and 47 μg/day, respectively. The mean relative standard error of the mean for the duplicate determinations was 5.0%.

Interference Tests.—Some biological materials may occasionally contain large amounts of iron or phosphorus (e.g., liver, milk) which are known to cause spectral interference at the 196.0 nm selenium resonance line (10–12), so experiments were performed to determine the per-

Table 5. Accuracy and precision of the method

Sample	No. of days[a]	Mean ± SD, ng Se/g dry wt	Day-to-day variation, RSD, %	Ref. value, ng Se/g dry wt
NBS Bovine Liver (SRM 1577)	16	1025 ± 87	8.5	1100 ± 100
IAEA Animal Muscle (H-4)	4	274 ± 35	12.9	280 ± 34
Infant formula powder (code 4)	9	89 ± 6	6.9	91[b]
Dried ground beef (code 5)	7	85 ± 8	9.7	83[b]
Wheat flour (code 2)	6	38 ± 5	12.1	43[b]
Dried human milk (code 3)	9	52 ± 4	7.3	56[b]
Pooled human serum	5	87 ± 5	6.0	89[c]

[a] One analysis per day.
[b] Median value obtained in intercalibration (see ref. 5).
[c] Median value obtained in intercalibration (see ref. 6).

missible levels of these matrix elements by adding increasing amounts of Fe(II) ($FeSO_4$) and Fe(III) ($FeCl_3$) to the water, diet, or the infant formula matrix, or $Ca_3(PO_4)_2$ to the water matrix after which a complete determination was performed.

The results of these experiments demonstrate that the interference limit for Fe(II) is approximately 3 times higher in the infant formula and over 5 times higher in the diet matrix compared with the water matrix (Table 6). Accordingly, the interference limit for Fe(III) is over 4 times higher in the infant formula matrix and over 6 times higher in the diet matrix compared with the water matrix. The ameliorating effect of other inorganic ions deriving from organic matrices on Fe(II) and Fe(III) interference is not known but most likely results from a reduction in iron extractability into the organic solvent. Phosphorus did not show any interference in this analytical system even at the P/Se ratio of 47 000 which can be explained by the inability of the PO_4^{-3} ions to be extracted into MIBK (Table 6). Based on these experiments, one may conclude that both the Fe/Se and the P/Se interference limits are high enough to allow determination of selenium in all foods and diets according to the concentration levels of these elements in 400 food commodities (19).

Detection Limit.—Figure 1 shows a recorder chart tracing of selenium analytical peaks for a dried whole milk sample containing 39 ng Se/g dry wt (5) together with 2.5 and 5.0 ng Se/mL standard solutions and analytical blanks. The detection limit defined as the mean ± 2 standard deviation of blanks was 0.6/ng Se/mL (n = 16), representing 3.1 ng Se/g dry wt for samples of 1.0 g.

Table 6. Maximum permissible ion/selenium (wt/wt) ratios for Fe(II), Fe(III), and P(V) in the determination of Se in different matrixes

Matrix	Matrix element	Added as	Limit ion/Se (wt/wt)
Water	Fe(II)	sulfate	500
Water	Fe(III)	chloride	750
Infant formula	Fe(II)	sulfate	1600
Diet	Fe(II)	sulfate	2700
Infant formula	Fe(III)	chloride	3300
Diet	Fe(III)	chloride	5000
Water	P(V)	$Ca_3(PO_4)_2$	47 000

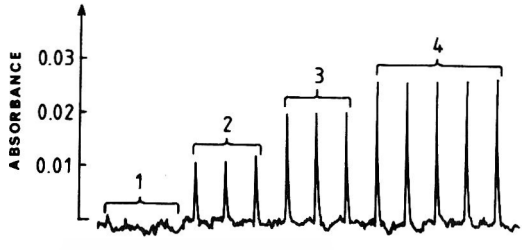

Figure 1. Recorder chart tracing of selenium analytical peaks. The replicate peaks in numerical order represent: 1, blanks; 2, 2.5; 3, 5.0 ng Se/mL standard solutions; and 4, 0.963 g sample of dried whole milk containing 39 ng Se/g.

Conclusions

The results of the collaborative study suggest that the tested ETAAS method without organic extraction is applicable to high selenium biological materials such as selenium nutritional supplements whose selenium content is ≥1 mg/kg dry wt. This method offers certain advantages over the other methods.

First, use of perchloric acid can be avoided in the digestion procedure because all selenium compounds need not be decomposed but only solubilized (9). Second, the analytical procedure is simple and rapid, allowing automated instrumental analysis and results in high sample throughput; approximately 20 duplicate determinations per day can be performed by one person.

Separation of selenium from interfering ions is necessary in the ETAAS determination of selenium in foods and diets whose selenium content is <1 mg/kg dry wt. The advantages of the tested ETAAS method employing the APDC-MIBK extraction system are (*1*) the practical detection limit is better than those of the spectrofluorometric or hydride generation methods; (*2*) sample throughput is higher compared with the spectrofluorometric methods, approximately 20 samples per day can be analyzed in duplicate by one person; (*3*) compared with the AAS methods based on hydride generation, the present method is more applicable for laboratories that do not have a separate instrument for selenium determinations, the graphite furnace need not be switched with the hydride generation apparatus; and (*4*) instrumental analysis is more convenient in the present method compared with either of the other methods because it is performed automatically.

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TOXICOLOGICAL METHODS

Screening of Fresh Water Fish Extracts for Enzyme-Inducing Substances by an Aryl Hydrocarbon Hydroxylase Induction Bioassay Technique

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A sensitive biological test to detect the presence of certain contaminants, such as highly toxic halogenated dioxins, dibenzofurans, and biphenyls in foods, was applied to extracts of fresh water fish that had been prepared by a food extraction-cleanup procedure developed by the Food and Drug Administration for pesticides and industrial chemicals. Aryl hydrocarbon hydroxylase (AHH) activity in a rat hepatoma cell line was used as the biological detection system for residues that induce enzyme activity. The induction of AHH activity by the extracts was compared with a standard AHH-induction curve for the most active compound known, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and results were computed as TCDD equivalents. Several dilutions of fish extracts were used to produce AHH-induction curves from which an optimal dose-response range was determined and used to estimate TCDD equivalents. Cleaned-up extracts of fish obtained from different water bodies in the United States were examined for AHH activity. The samples which had low levels of polyhalogenated contaminants produced low biological activity, while a higher activity was obtained from fish that contained higher levels of polyhalogenated contaminants. The results suggest that the fish extracts can be screened for AHH inducers before chemical analysis.

The detection, identification, and quantitation of hazardous chemicals in food are essential for safety assessment, and results of such assessments are needed for regulatory purposes. Detailed analyses of food samples for all possible hazardous chemicals are often tedious and time-consuming; therefore a highly specific biological test that reflects a toxicologic end point may be useful as an initial screening process for food samples that are suspected of containing chemical contaminants.

Of particular interest are toxic and potent enzyme-inducing chemicals such as some of the polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs). Their presence in the environment has been documented (1). Because of their properties such as chemical stability, solubility in lipids, and mobility, they are potential food contaminants. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and some of its congeners induce the activity of a variety of enzymes including aryl hydrocarbon hydroxylase (AHH) (2, 3). PCBs and PCDFs have been screened for their ability to induce AHH (3), and there is a correlation between the toxicity of certain classes of polyhalogenated organic substances and their ability to induce AHH (2, 3).

The primary objective of the present study was to determine if the cell culture-AHH induction test could be used to screen specially prepared fish extracts for enzyme-inducing residues of industrial origin, especially those belonging to the PCB, PCDD, and PCDF families. Food extraction-cleanup procedures developed for pesticides and industrial chemicals were used to prepare the extracts. The results of screening extracts by the AHH assay and correlation with some previous chemical analyses of the fish by gas-liquid chromatography (GLC) are reported.

Experimental

Fish Samples

The types of fish and locations where they were collected are listed in Table 1. Only the edible portion of the fish was used. Samples were supplied as frozen whole fish, fillets, or ground fillets.

Sample Preparation for Bioassay

Preparation of silica gel column.—To a 22 mm id chromatographic column equipped with a coarse fritted glass dish and a Teflon stopcock, 50 mL petroleum ether was added. Twenty g hot silica gel (60–200 mesh, J. T. Baker Chemical Co., Phillipsburg, NJ, No. 5-3405), which had been activated overnight at 130°C, was poured into the

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Table 1. Analysis of fish samples by GLC multiresidue method and AHH-induction bioassay

Fish sample (collection location)	Sample No.	GLC assay: Chemicals[a]	GLC assay: Concn, ppm	Bioassay: TCDD equiv., pg TCDD/g fish (ppt)
Flathead catfish (Missouri River)	1a	Chlordane[b]	0.1	29
		PCB (Aroclors 1254 & 1260)	0.5	
		Dieldrin[b]	0.014–0.12	
	1b	Same composite as 1a		—[c]
	1c	Same as 1a, but fortified with TCDD equiv. to 21.7 ppt		50
Bass (lake near Hattiesburg, MS)	2a	PCB	0.2	27
		PCA	0.1	
		TCA isomers:		
		2,3,5,6-	0.018	
		2,3,4,6-	0.009	
		2,3,4,5-	0.014	
		Chlorinated DPE (7–10 chlorines)	0.002–0.06	
		Methoxy-substituted Cl_9DPE	0.02	
	2b	Same as 2a, but fortified with 2,3,4,7,8-PCDF equiv. to 3121 ppt		159
Catfish (Saginaw Bay, MI)[d]	3	TCDD	0.035[e]	251
		PCB (Aroclor 1254)	10	
		p,p'-DDE	1.6	
Carp (Sheboygan River, WI, above Kohler Dam)	4	PCB (Aroclor 1242: 1254 ratio 75:25)	370	1877
Carp (Sheboygan River, WI, Kiwanis Park)	5	PCB (Aroclor 1242:1254 ratio 60:40)	50	56

[a] Abbreviations: PCB; polychlorinated biphenyls; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCA, pentachloroanisole; TCA, tetrachloroanisole; DPE, diphenyl ether; PCDF, pentachlorodibenzofuran; DDE, dichlorodiphenyldichloroethylene.
[b] This chemical is not an AHH inducer (3).
[c] At or below the quantitation limit of 25 pg TCDD.
[d] The GLC assay values were obtained by analysis of the cleaned-up extract by electron capture GLC.
[e] ppb.

petroleum ether through a funnel, and ca ½ in. layer of Na_2SO_4 was added. The petroleum ether was allowed to elute until the liquid level was ca 3 mm above the Na_2SO_4.

Extraction and cleanup.—The extraction procedure for high-moisture products in a multiresidue method for nonfatty foods (4) was used, except that the sample weight was 30 g and the factor *T* [(mL water in sample) + (mL acetonitrile added) − (correction in mL for volume contraction)] was 220 mL. The petroleum ether extract obtained through this method was concentrated to ca 5 mL in a Kuderna-Danish (K-D) concentrator. The concentrate was applied on the silica gel column and eluted with petroleum ether. The first 50 mL eluate was discarded and the next 250 mL was collected. This eluate was transferred to a K-D concentrator and concentrated to ca 5 mL. Isooctane (0.5 mL) was added to the K-D tube and the solution was evaporated to ca 0.5 mL, using a micro-Snyder column. The final volume was adjusted to 1 mL with isooctane. Whenever required, known active compounds were added at this point.

Bioassay Procedure

A rat hepatoma cell line, H-4-11-E, was used for these studies. The experimental conditions, medium composition, and measurement of AHH specific activity and protein content have previously been published (3, 5, 6). Eight serial dilutions of the 1 mL samples from silica gel cleanup were prepared to range from 100 to 0.03% (v/v) of the original sample, and 0.1 mL of each sample extract or dilution was added to triplicate plates containing cell monolayers at 24 h growth. Concurrently, TCDD was added to another series of plates at 10 different concentrations ranging from 5 to 1000 pg/plate for the standard TCDD-AHH activity curve (Figure 1). Control cells received isooctane solvent alone at 0.1 mL/plate. Cells were collected after 72 h of incubation at 37°C in 5% CO_2–95% air and the specific AHH activity was computed as pmol

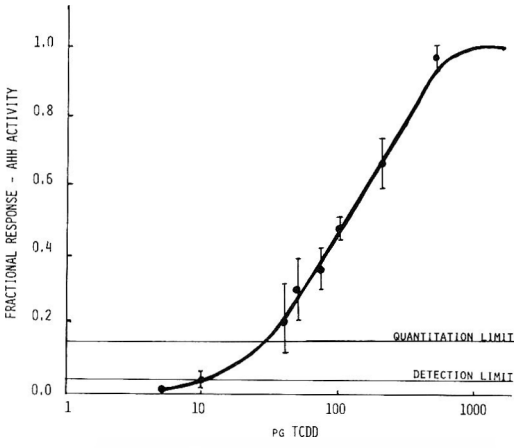

Figure 1. TCDD dose response curve. Mean fractional response and standard errors of AHH activity are plotted as function of TCDD concentration (pg/plate) for 4 experiments.

3-hydroxybenzo[*a*]pyrene per mg protein per min. The TCDD-AHH response curve was used to determine activity induced by the same extract and the dilutions of the extract, and the activity was equated to the relative amount of TCDD necessary to achieve similar induction. The limit of detection was 10 pg TCDD/plate. However, the quantitative or linear portion of the curve, 25–500 pg TCDD, was used to estimate TCDD equivalents for fish extracts or dilutions.

Multiresidue Analysis

The fish samples were analyzed for pesticides and early eluting residues by the *Pesticide Analytical Manual* multiresidue method for nonfatty foods (4). PCDDs, PCDFs, and chlorinated methoxybiphenyls were determined as described by Phillipson and Puma (7).

Results and Discussion

The primary objective of the extraction-cleanup procedure was to provide a fish extract that would contain all the PCBs, PCDDs, and PCDFs present as contaminants in the sample, while removing any lipids or other natural components of fish tissue that might interfere in the bioassay or prevent concentration of the toxic compounds of interest to an appropriate level in the final solution.

We tested the extraction-cleanup procedure described in the Experimental section for recovery of several commercial PCBs (Aroclors), 3 planar PCB congeners (3,3′,4,4′-Cl_4, 3,3′,4,4′,5-Cl_5, and 3,3′,4,4′,5,5′-Cl_6), PCDFs at all chlorination levels from trichloro through octachloro, and selected PCDDs containing 3, 4, 6, 7, and 8 chlorine atoms. Ground fillets of ocean perch were used as the substrate for the recovery studies. All of these chemicals were quantitatively recovered in the silica gel eluate fraction collected for the enzyme induction test. Of other compounds included in recovery studies with fish, chlorinated naphthalenes were found to elute from the silica gel column before the appropriate fraction is collected, and, as previously reported (7), heptachlor epoxide, octachlor epoxide, and chlorinated methoxybiphenyls (several Cl_3-, Cl_4-, and Cl_5-isomers) are retained on the column. Thus, the extraction-cleanup procedure may have excluded unknown AHH inducers in the samples or it may have admitted others of which we were not aware.

We observed during the analyses of fish (rather than in systematic recovery studies) that *p,p′*-DDE, pentachloroanisole, and tetrachloroanisole are recovered (at undetermined levels) in the appropriate fraction and that *p,p′*-DDT, chlordane, and phthalate esters are retained on the silica gel column beyond this fraction. In general, we would expect that polar residues would be eliminated by the procedure and that nonpolar organohalogen compounds, such as brominated biphenyls, chlorinated benzenes, aldrin, and mirex, either would be recovered in the appropriate fraction or would elute with the chlorinated naphthalenes.

Table 1 summarizes the results of multiresidue analyses of the fish extracts and AHH induction of the silica gel cleaned-up fraction of the extracts. Not all of the chemicals found in the fish extracts would necessarily be present in the AHH test fractions because of the silica gel cleanup.

The bioassay results are expressed as TCDD equivalents in pg per g of fish (parts per trillion). The detection limit for fish samples is the equivalent of 10 pg TCDD/0.1 mL fish extract/plate or the AHH activity that was 3 times that of solvent control. The lower quantitation limit is 25 pg TCDD/0.1 mL fish extract/plate. Maximum induction occurred at a TCDD concentration ≥500 pg/plate. The induction of AHH activity achieved by the fish extracts or extract dilutions that fell between 25 and 500 pg TCDD quantitation (the linear portion of the TCDD standard curve) was used to compute TCDD equivalents.

Samples 1a and 1b, composites from 2 flathead catfish collected from the Missouri River 310–460 miles upstream from St. Louis, induced a very

low AHH activity. This observation is consistent with the chemical analysis of these samples which showed that they contained low-level residues of PCBs (<0.5 ppm) with a mixture of Aroclor 1254 and Aroclor 1260. Sample 1c, of the same composite as 1a and 1b, induced AHH activity that reflected the additive effect of the 21.7 ppt TCDD addition.

The bass, Sample 2a, was collected from a lake near Hattiesburg, MS, shortly after an overflow of a holding pond containing waste pentachlorophenol from a wood treatment plant. However, no PCDDs and PCDFs were found in this sample. The multiresidue extract produced a very low level of AHH-inducing substances, which reflects the low level of contamination in this fish sample.

Sample 2b, prepared from the same fish described above, was fortified with 3.1 ppb 2,3,4,7,8-PCDF. The increased AHH activity indicated the inducing effect of this congener in the extract.

Sample 3, obtained from catfish caught in Saginaw Bay, MI, contained 35 ppt TCDD (D. Firestone, Food and Drug Administration, personal communication, 1981); however, the biological activity of 251 ppt TCDD equivalents indicates the presence of inducers in addition to TCDD. The limited amount of this fish sample did not allow for multiresidue analysis. However, the cleaned-up sample solution prepared for the enzyme induction study was examined by electron capture GLC to estimate the levels of electron-capturing residues present. The chromatogram contained a peak at the retention time of *p,p'*-DDE superimposed on a chromatographic pattern typical of PCBs having the composition of Aroclor 1254. No attempt was made to separate the residues. However, the residue levels were estimated to be about 10 ppm PCB and no more than 1.6 ppm *p,p'*-DDE. The effect of *p,p'*-DDE alone on AHH activity is not known.

Analysis by the multiresidue method revealed the presence of 370 ppm PCBs with an Aroclor 1242/Aroclor 1254 ratio of 75/25 in carp (Sample 4) collected from the Sheboygan River upstream from Kohler Dam in Wisconsin. An unusually high level of AHH activity was obtained, compared with other samples tested by the AHH-induction technique. Another carp (Sample 5), caught in Kiwanis Park, WI, contained PCBs at 50 ppm with an Aroclor 1242/Aroclor 1254 ratio of 60/40, and the biological activity was low. No chlorinated dioxins and furans were present in either Sample 4 or 5. The high activity of Sample 4 is attributed to substance(s) yet to be identified.

All fish samples analyzed in this study contained PCBs. Other studies have shown that several polyhalogenated biphenyl analogs and their mixtures induce AHH activity (2); however, in our study it is not possible to attribute AHH activity solely to PCBs in fish extracts because other compounds may also have contributed to the activity.

The bioassay does not specifically identify or classify the reactive substances in food; however, as this study shows, it is useful as a screen for the presence of substances that induce AHH activity. The level of induction by such substances in comparison with that by TCDD allows quantitation of unknown inducers relative to TCDD, the most potent inducer known.

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Atomic Absorption Determination of Serum Copper: Collaborative Study

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Preliminary to conducting a collaborative study on a method for copper in serum, methods used by a selected group of laboratories were surveyed. The responding laboratories were supplied with a Youden pair of bovine serum samples and requested to use their current method for serum copper. Results of the analyses and the methods used were evaluated; hypotheses were developed in our laboratory to explain some of the interlaboratory variation. For the AAS method chosen, each of 12 collaborating laboratories analyzed one blind duplicate and 2 Youden pair of serum samples. A commercially available external control serum with a certified level of copper and a 1000 mg copper/L standard were also submitted. The method requires the serum to be diluted 1 + 1 with distilled water and the standards to be diluted with 10% glycerin to approximate the viscosity of the diluted serum. The intralaboratory coefficients of variation (CV) ranged from 2.24 to 4.40% and the interlaboratory CV ranged from 2.56% to 6.05%. The method has been adopted official first action.

The relationship of dietary copper, molybdenum, and sulfate to animal nutrition has been well documented by Underwood (1). The syndrome caused by high dietary copper and/or low molybdenum intake as well as several industrial and naturally occurring sources of toxic levels of copper has been described by Buck et al. (2). A survey of methods conducted by our laboratory of copper analysis in serum indicates the need for an official method of copper analysis (3).

The method chosen for use in this collaborative study is a slight modification of the procedure for serum copper and zinc recorded in the Perkin-Elmer book of methods (4). The method was submitted to 12 collaborating laboratories along with one practice serum, 2 Youden pair of samples, one blind duplicate pair of samples, a commercially available external control serum (Precilip®), and a 1000 mg copper/L standard. The laboratories were requested to run each serum in duplicate. Mohr pipets were used to pipet the serum samples to ensure an accurate volume.

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Copper in Serum
Atomic Absorption
Spectrophotometric Method
Official First Action

25.D01 *Principle*

Samples are dild 1 + 1 with H_2O, and Cu is detd by AAS using std solns prepd in 10% glycerol.

25.D02 *Apparatus and Reagents*

(a) *Atomic absorption spectrophotometer.*—Equipped with nebulizer and air-C_2H_2 burner head. Monitor performance by assuring that 4.0 mg/L std produces response ≥0.200 absorbance unit.

(b) *External control.*—Precilip, Cat. No. 125067 (Bio-Dynamics/bmc, 9115 Hague Rd, Indianapolis, IN 46250), or equiv. with established value for Cu. Dil. according to label.

(c) *Glycerol USP.*—10% (v/v) aq. soln.

(d) *Copper std solns.*—(*1*) *Stock std soln.*—1000 mg/L. Dissolve 1.000 g Cu metal in min. vol. of HNO_3-H_2O (1 + 1). Dil. to 1000 mL with 1% HNO_3. (*2*) *Intermediate std soln.*—100 mg/L. Dil. 10 mL stock std soln to 100 mL with H_2O. (*3*) *Working std solns.*—Dil. 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0 mL intermediate std soln to 100 mL with 10% glycerol to give std solns contg 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0 mg Cu/L.

25.D03 *Sample Preparation*

Rinse all glassware used with 2N HCl. Mix samples thoroly before pipetting. Using Mohr pipet, transfer 1.0 mL serum and 1.0 mL Precilip (external control) to sep. test tubes. Add 1.0 mL H_2O to each and mix 5 s on vortex mixer or cap tubes and shake 10 s. Use 1 external control for each 10 samples or fraction thereof.

Table 1. Collaborative data[a,b] for AAS determination of serum copper

Lab.	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
1	190	195	207	207	66	66	62	62	62	66	66	66
2	211	196	211	211	72	67	67	67	67	67	67	67
3[c]	174	176	202	200	64	66	58	56	58	58	66	62
4	194	202	214	210	68	68	64	64	64	68	72	68
5	192	208	200	220	74	74	70	70	62	70	78	74
6[c]	250	245	265	270	65	75	80	85	85	95	90	95
7	212	214	208	200	64	58	62	56	68	64	76	72
8	200	204	220	216	66	68	66	68	64	66	66	72
9	200	196	212	212	72	68	68	68	70	70	70	70
10	200	200	210	208	65	65	63	65	65	65	68	70
11	223	216	209	213	66	68	60	60	59	60	61	70

[a] Units are μg copper/100 mL serum.
[b] Results of duplicate analysis on each sample.
[c] Results of outlying laboratory excluded by ranking.

25.D04 ***Determination***

Analyze by AAS using the following conditions: wavelength 324.7 nm; slit 0.7 nm; flame air–C_2H_2 (lean-blue). Aspirate series of working std solns, external control soln, and sample dilns. Repeat analysis if Cu value in external control soln is not within accepted range. Prep. std curve of concn, mg Cu/L, vs A, and det. concn of sample. Multiply result by 200 to account for sample diln and to convert result to μg Cu/100 mL.

Results and Discussion

Complete results were received from all 12 of the participating laboratories. However, one set of results was not returned in time for the statistical review (Table 1). Three laboratories made slight modifications of the prescribed procedure: One laboratory used an Oxford micropipetter instead of a Mohr pipet. One laboratory used volumetric pipets to transfer serum. Work in our laboratory indicates that, probably due to the difference in viscosity of serum vs water, a full measure of serum is not delivered with a volumetric pipet. One laboratory added glycerol to the standards instead of making up a 10% glycerol solution. It is the consensus in our laboratory that since glycerol is not immediately miscible with water, it is preferable to dilute the standards with a solution of glycerol in water.

Most of the laboratories submitted standard curves with their analytical results. A standard curve is not necessary for calculating results in all cases because many instruments have a direct concentration readout or linear regression device. The curve was requested to ensure the linearity of the individual instrument over the concentration range of the samples.

Samples 1 and 2 and Samples 5 and 6 were Youden pairs, while Samples 3 and 4 were blind duplicates. Statistical analysis was performed according to the AOAC statistical manual (5). Laboratories 3 and 6 were outliers, based on the Youden rank test. The remaining results were used to calculate the means, standard deviations, and coefficients of variation recorded in Table 2. Because of excellent statistical agreement, no further statistical comparison was done on the blind duplicates even though blind duplicates can give a better indication of the repeatability of a method than duplicates of all samples.

The low standard deviations obtained can be explained by the relative ease of copper analyses by AA, the simplicity of the method, the inclu-

Table 2. Statistical data from collaborative study

	Sample Number					
	1	2	3	4	5	6
Average (μg/100 mL)	203	210	68	65	65	70
Repeatability	6	5	2	2	3	3
CV, %	2.97	2.54	3.22	2.42	3.90	4.40
Reproducibility	9	5	4	4	3	4
CV, %	4.60	2.56	5.81	6.05	5.07	5.91

sion of a practice sample which encourages the collaborator to become familiar with the method before processing the collaborative samples, and most important, the use of an external control with a specified range of acceptable values. Two of the 3 laboratories which did not obtain results within this specified range were outliers. This supported the statistical basis for rejection of these results.

No estimate of accuracy can be made from this study because no spiked samples were included. Copper is known to be bound in serum, so the addition of the free metal as a check on recovery is not a true representation of copper in serum. Copper in bound form is available commercially and could have been used for spiking had the cost not been prohibitive.

Two laboratories were requested to and did supply data from ICP and carbon furnace methodology. The data are being evaluated for possible use in a future collaborative study.

Recommendation

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

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

The recommendation of the Associate Referee was approved by the General Referee and Committee G and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* **66**, 405, 437 (1983).

ANTIOXIDANTS

Reverse Phase High Pressure Liquid Chromatography and Fluorescence Detection of Ethoxyquin in Milk

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A high pressure liquid chromatographic (HPLC) method has been developed for the determination of ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) in milk. Milk solids are precipitated by adding acetonitrile, and the water-acetonitrile supernate is washed with hexane to remove fat. Addition of sodium chloride causes the water-acetonitrile solution to separate into an aqueous phase and an acetonitrile phase, thus separating ethoxyquin from most water-soluble impurities. A large volume of water is then added to the acetonitrile layer and ethoxyquin is partitioned into hexane, which is removed at reduced pressure. The residue is dissolved in the mobile phase and analyzed on a 4.6 mm id × 250 mm Ultrasphere ODS column using fluorescence detection (excitation 230 nm; 418 nm cutoff filter). Water-acetonitrile with a diethylamine-acetic acid buffer is the mobile phase. Recoveries from samples fortified at 1, 5, and 10 ppb averaged 78% with a coefficient of variation of 5.0%. Low levels (<1 ppb) of apparent ethoxyquin were found in commercial milk samples that were analyzed by using the method.

In the United States, ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) is used as an antioxidant in animal feeds at levels up to 150 ppm. Consequently, tolerances are specified for ethoxyquin residues in edible animal tissues (0.5–5 ppm), poultry and eggs (0.5 ppm), and milk (zero). Although there is an official AOAC method for determining ethoxyquin in animal tissues and eggs (1), none exists for determining ethoxyquin in milk, nor could an adequate method be found in the literature. The purpose of this study, therefore, was to develop a reliable method for determining ethoxyquin in milk.

Dunkley et al. (2) found 7 ppb ethoxyquin in the milk of cows fed a diet containing 150 ppm ethoxyquin. They also noted an unidentified substance in the milk that interfered with the determination of ethoxyquin. Thus, a more reliable method capable of detecting very low levels of ethoxyquin was needed. Fluorescence methods are highly sensitive and have been widely used for determining ethoxyquin (1–4), but these methods lack specificity. More recently, gas-liquid chromatography has been used (5), but this technique is not well suited to the determination of ethoxyquin because of the thermal instability of this compound. High pressure liquid chromatography (HPLC) is a more suitable technique and has, in fact, been used with ultraviolet (UV) detection to determine high levels (about 100 ppm) of ethoxyquin in spices (6).

By combining a reverse phase HPLC separation with fluorescence detection, a highly sensitive and specific method has been developed for determining ethoxyquin in milk. Milk samples are cleaned up by solvent extraction and concentrated before chromatographic analysis. The method is designed to operate in the 1–10 ppb range.

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METHOD

Apparatus

(a) *Liquid chromatograph.*—Spectra-Physics Model 3500B operated at ambient temperature (Spectra-Physics, Piscataway, NJ 08854).

(b) *Injector.*—Rheodyne Model 7125 sample injection valve fitted with 20 μL sample loop (Rheodyne Inc., Berkeley, CA 94710).

(c) *Column.*—4.6 mm id × 250 mm stainless steel, packed with Ultrasphere ODS (ca 10 000–15 000 theoretical plates) (Altex Scientific, Inc., Berkeley, CA 94710).

(d) *Detectors.*—Schoeffel Model 970 fluorescence detector (Kratos, Inc., Westwood, NJ 07675). Range 0.01–1 μA full scale; 5 μL flow cell. Excitation wavelength 230 nm, 418 nm cutoff filter, time constant 2 s. Schoeffel Model 770 UV detector (Kratos) used to obtain chromatogram in Figure 1. Range 0.1–2 absorbance units full scale (AUFS); 8 μL flow cell, time constant 2 s. Chromatograms were recorded on 10 mV strip chart recorder.

(e) *Centrifuge.*—Sorvall RC-3 (E.I. duPont de Nemours & Co., Wilmington, DE 19898) or cen-

trifuge capable of relative centrifugal force of 1000.

(f) *Flash evaporator.*—Used with 35°C water bath (Buchler Instruments, Inc., Fort Lee, NJ 07024).

Reagents

(a) *Solvents.*—Distilled-in-glass grade acetonitrile (UV), hexane, methanol, and toluene (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

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

(c) *Presaturated solvents.*—Acetonitrile and water, both saturated with hexane, and hexane saturated with water. Prepare as needed.

(d) *Silanizing solution.*—10% (by volume) dimethyldichlorosilane (Applied Science Division, Milton Roy Laboratory Group, State College, PA 16801) in toluene.

(e) *Buffer solution.*—Dissolve 0.6 mL acetic acid in 500 mL water. Add 1 mL diethylamine (reagent grade, Fisher Scientific Co., Pittsburgh, PA 15219) and dilute to 1 L with water.

(f) *Mobile phase.*—20% (by volume) buffer solution in acetonitrile.

(g) *Ethoxyquin.*—Technical grade (Pflaltz and Bauer, Inc., Stamford, CT 06902). Determine purity (6).

(h) *Standard solutions.—Stock solution 1.*—10 μg/mL. Accurately weigh 0.1 g ethoxyquin and transfer to 100 mL volumetric flask; dissolve and dilute to volume with acetonitrile. Pipet 1 mL into second 100 mL volumetric flask containing 20 mL buffer solution and dilute to volume with acetonitrile. *Stock solution 2.*—250 ng/mL. Pipet 5 mL stock solution 1 into 200 mL volumetric flask and dilute to volume with mobile phase. *Working solutions.*—5, 20, 35, and 50 ng/mL. Pipet 1, 4, 7, and 10 mL stock solution 2 into 50 mL volumetric flasks and dilute to volume with mobile phase. Store all standard solutions in dark.

Determination

Glassware silanization.—Rinse clean glassware with 0.1N NaOH, and then with water until neutral. Rinse with 0.1N HCl, and then with water until neutral. Rinse with methanol, and then toluene. Fill item with silanizing solution; let stand 5 min. Rinse with toluene, and then methanol; let air-dry. Silanizing solution may be reused. Glassware may be reused without repeating silanization process provided corrosive cleaning solution (e.g., chromic acid) is not used.

Extraction.—Weigh 50 g milk into 250 mL screw-cap polyethylene centrifuge bottle. Add 75 mL acetonitrile and shake 1 min. Warm to room temperature in 35°C water bath. Centrifuge 10 min at 2000 rpm. Decant supernate into 500 mL separatory funnel and wash with 10 mL presaturated hexane. Transfer lower layer to second 500 mL separatory funnel. Back-extract hexane layer with 5 mL presaturated acetonitrile; add acetonitrile (lower) layer to second separatory funnel. Discard hexane layer; rinse separatory funnel with ca 5 mL acetonitrile. Discard rinsings but retain separatory funnel. Add 3 g NaCl and 1 mL 3N NaOH to water–acetonitrile solution in second separatory funnel; shake vigorously 1 min to dissolve salt. Let solution separate into 2 layers. Discard aqueous (lower) layer. Add 150 mL presaturated water and 1 mL 3N NaOH to acetonitrile layer and extract with 50 mL presaturated hexane by shaking gently 1 min. Let layers separate 10 min. Drain water–acetonitrile (lower) layer into first separatory funnel and repeat extraction with 25 mL presaturated hexane. After layers have separated, discard lower layer. (If emulsion persists in either hexane layer, add 1–2 mL saturated NaCl. As emulsion breaks, drain off lower layer. Add more saturated NaCl if necessary.) Wash each hexane layer with 15 mL presaturated water; discard water layers. Combine hexane layers in 250 mL round-bottom flask. Rinse each separatory funnel with 10 mL acetonitrile and add rinse to flask. Concentrate to 1–2 mL on rotary evaporator using 35°C water bath. With disposable pipet, transfer solution to 5 mL volumetric flask containing 1 mL buffer solution. Rinse round-bottom flask with 1–2 mL acetonitrile; transfer to volumetric flask. Dilute to volume with acetonitrile.

Chromatography.—Sparge mobile phase with helium 15 min. Maintaining sparge throughout analysis, pump ca 30 column volumes of mobile phase through column at 1.5–2 mL/min. Then adjust flow rate to 1.0 mL/min. Set fluorescence detector sensitivity to 5.0 and range to 0.2 μA full scale. Inject 20 μL sample solution and 4 working solutions. Adjust range if necessary. If sample peak is larger than peak for most concentrated working solution, accurately dilute sample solution.

Calculation

Determine peak heights for sample and working solutions. Plot peak height vs con-

centration for working solutions and determine slope (S) and intercept (I) of line by performing linear least squares analysis. Calculate concentration of ethoxyquin in milk sample as follows:

$$\text{Ethoxyquin, ppb} = ((H - I)/S) \times (5/W) \times D$$

where H = peak height of sample; W = weight of sample, g; and D = dilution factor if sample solution is diluted.

Results and Discussion

Analysis of amines by reverse phase HPLC often results in broad, asymmetric peaks with long retention times. Such behavior has been attributed to strong interactions between the amine functional group and unreacted silanol groups on the packing material. Addition of alkylamines (7) or ammonium compounds (6, 8) to the mobile phase minimizes these interactions. In the present study, a diethylamine–acetic acid buffer was added to the mobile phase, resulting in sharp, symmetrical peaks for ethoxyquin. About 30 column volumes of mobile phase had to be passed through the column before reproducible responses were observed for dilute (< 100 ng/mL) ethoxyquin solutions. No other columns were tried.

Removing dissolved oxygen from the mobile phase by sparging with helium increased sensitivity. Ethoxyquin peak heights almost doubled when oxygen was excluded, although column efficiency was unaffected. Perhaps dissolved oxygen reacts with the injected ethoxyquin or partially quenches its fluorescence.

Low levels of ethoxyquin can be detected because of its fluorescent nature. With the conditions specified for this method, a 1 ng/mL solution gave a response 20 times the noise level of the detector. Since a milk sample containing 100 ppt ethoxyquin would yield a sample solution of 1 ng/mL, 100 ppt is considered to be the lowest level that could reliably be quantitated by the method. A comparison of fluorescence detection with UV detection for a 5 ppb spiked milk sample is shown in Figure 1.

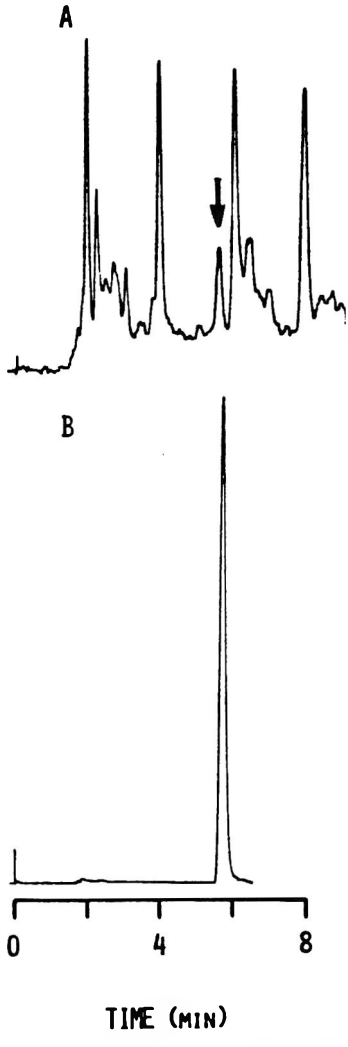

Figure 1. Chromatograms of 5 ppb spiked milk sample. A, UV detection, 230 nm, 0.01 AUFS, arrow indicates ethoxyquin peak; B, fluorescence detection, 0.2 μA full scale.

During the early part of the study, replicate ethoxyquin standard solutions gave widely different responses when unsilanized glassware was used; for example, when a 50 ng/mL standard was analyzed, peak heights of 3.59, 4.12, 3.77, 3.37, 3.93, and 1.99 cm were obtained. This phenomenon was attributed to adsorption of ethoxyquin on the surface of the glassware, since good reproducibility was observed when silanized glassware was used to prepare standards. Use of silanized glassware greatly improved the recovery of ethoxyquin from spiked reagent blanks, from 0 to 10% with unsilanized glassware and from 30 to 60% with silanized glassware. For spiked milk samples, however, recoveries averaged about 80% regardless of which type of glassware was used. However, precision was improved by a factor of 3 with silanized glassware.

Preparation of milk samples for HPLC involves a series of solvent extractions. The first step of the cleanup, addition of acetonitrile,

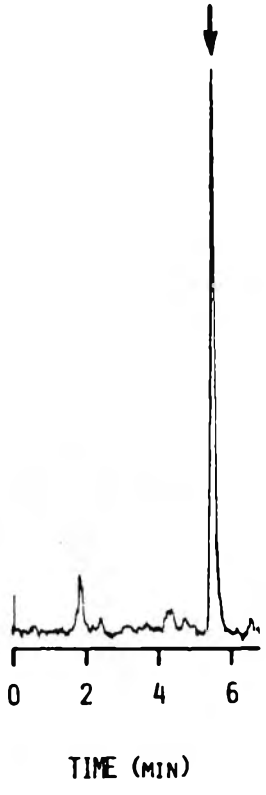

Figure 2. Chromatogram of 1 ppb spiked milk sample (0.05 μA full scale), arrow indicates ethoxyquin peak.

causes precipitation of milk solids, which are removed by centrifugation. Fatty impurities are removed by washing the water–acetonitrile supernate with hexane; back-extraction of the hexane layer with acetonitrile recovers any ethoxyquin lost by this step. Addition of sodium chloride and base causes the solution to separate into an aqueous phase and an organic (acetonitrile) phase. Ethoxyquin, which is hydrophobic in basic solution, migrates to the organic phase; proteins and other hydrophilic impurities are retained in the aqueous phase. The acetonitrile layer is fairly clean, but it is unsuitable for HPLC since it contains appreciable amounts of sodium chloride, which corrodes stainless steel. To remove ethoxyquin from this solution, a large volume of water is added and ethoxyquin is partitioned into hexane. The hexane layer is collected in a round-bottom flask and the solvent is removed with a rotary evaporator. Enough acetonitrile is added to the flask so that all of the hexane can be removed without evaporating to dryness, thus preventing loss of ethoxyquin through evaporation. The concentrate is diluted to 5 mL with the mobile phase. The column efficiency is higher if the mobile phase, rather than pure acetonitrile, is used as the solvent.

Using the described method, recoveries of ethoxyquin from milk were determined at 1, 5, and 10 ppb. A sample was prepared by spiking 50 g milk in a polyethylene bottle with 1 mL stock solution and shaking. Twenty samples at each level were prepared and analyzed. Milk blanks were also analyzed; responses, if any, were subtracted before calculating recoveries. The average percent recoveries at 1, 5, and 10 ppb were 79 (coefficient of variation (CV) 5.6), 76 (CV 4.8), and 79 (CV 3.9), respectively. The average recovery for all 60 samples was 78% with a CV of 5.0%. Figure 2 shows a typical chromatogram for a 1 ppb spiked milk sample.

The results indicate that about 20% of the added ethoxyquin was not recovered by the method. To determine at what point(s) ethoxyquin was lost, a series of milk blanks were prepared and spiked at different steps throughout the cleanup procedure. Virtually all of the 20% loss occurred during the initial extraction of the milk with acetonitrile. The lost ethoxyquin could not be recovered by further extraction of the precipitated milk solids.

Recoveries of ethoxyquin from spiked reagent blanks were only 30–60% (even using silanized glassware), compared to about 80% from spiked milk samples. Because of this discrepancy, we were concerned that high incurred residues of ethoxyquin in the milk samples being spiked could be causing the recovery values to appear higher than was actually the case. Consequently, some recoveries were determined using milk from cows fed a diet free of ethoxyquin. Using ethoxyquin-free milk, recoveries averaged 79%, compared to 77% with milk from commercial sources. These data demonstrate that incurred residues are not responsible for the difference in recovery from milk and from solvent. A possible explanation for the lower recovery from solvent is adsorption of ethoxyquin on incompletely silanized glassware. For milk, coating of the glassware by some component (e.g., fat) probably prevents loss by adsorption.

During method development, several milk blanks were analyzed that contained 1–2 ppb apparent ethoxyquin. After recovery studies were completed, samples of 6 different commercial brands of milk were purchased and analyzed; these samples contained 100–200 ppt apparent ethoxyquin. It should be emphasized that these values are estimates, since the method

was not documented below 1 ppb. The amount of ethoxyquin residues in milk is apparently small, based on the results of this limited study.

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ALCOHOLIC BEVERAGES

Proof Determination of Liqueurs and Alcoholic Dairy Products: Collaborative Study

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A method is described for determining the ethanol concentration of liqueur-type products containing dissolved solids. The sample is distilled in an all-glass still equipped with spherical joints; the proof of the distillate, diluted to known volume, is measured by a Mettler/Paar density meter. Ten collaborators determined proof of 6 different synthetic liqueurs containing ethanol–water–sugar mixtures and 2 alcoholic dairy products. Proof of the samples varied from 33 to 92°. Standard deviations and coefficients of variation for this method varied from 0.069 to 0.278 and from 0.20° to 0.36°, respectively. The method has been adopted official first action.

Dissolved solids in alcoholic products such as liqueurs, wines, and certain types of whisky, brandy, and rum interfere with standard proofing procedures using hydrometer, pycnometer, refractometer, and/or density meter. Therefore, these products must be distilled to obtain an accurate measurement of the ethanol concentration in the distillate.

The present AOAC procedure (1) for determining the proof of liqueurs is not used by members of the distilling industry because of the time and special techniques required. This procedure specifies distilling a 50 or 100 mL sample in a 500 mL distilling flask equipped with an Iowa-type bulb and a Liebig condenser. Distillate is collected in a special 50 or 100 mL volumetric-type flask which is used as a pycnometer. Accuracy of results obtained by this method has not recently been determined by an AOAC collaborative study.

One proofing method used by many U.S. plants producing liqueurs and similar products containing dissolved solids involves the use of a Jaulmes still, manufactured in France (2). This macro procedure requires a 500 mL sample. After the distillate is collected and diluted to volume, its proof is normally determined by a hydrometer. A major advantage of using a Jaulmes still is that the 500 mL sample can be distilled in 7–14 min vs usually more than 1 h by other distillation methods. Disadvantages of this method are 1) the need to add a correction factor of 0.1–0.3° to the proof result because alcohol recovery is not quantitative, and the correction factor must be determined for each still with ethanol standards in the same proof range as the samples tested; 2) the need for a 500 mL sample; and 3) the cost of the still ($10 000).

This collaborative study was initiated to develop a simple, accurate proofing procedure for alcoholic products such as liqueurs and alcoholic dairy products which contain dissolved solids such as sugar, protein, and fat; and to determine the accuracy of measuring the proof of these products with a method using current state-of-the-art distillation and proofing techniques. The method being evaluated is currently used in Seagram's laboratories to determine the proof of all alcoholic products containing dissolved solids which restrict direct proofing. Products proofed by this method include all liqueur-type products, whisky, brandy, rum, and wine. In this method, a 50 mL sample is distilled in an all-glass still (Seagram's Liqueur Still) equipped with spherical glass joints. Distillate is collected, diluted to volume, and proofed by a Mettler/Paar density meter using the AOAC method (3). Advantages of this method are that only 50 mL sample is distilled which reduces product losses and the amount of ethanol and sugar discharged to the wastewater system which is taxed on its BOD concentration, and that it uses a density meter to

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measure proof, which is easy to operate, requires less analysis time and smaller sample volumes, and gives more reproducible results. The disadvantage of this procedure is cost of the density meter ($13 500).

Collaborative Study

Ten collaborators received 6 different synthetic liqueurs containing ethanol–water–sugar mixtures, 2 alcoholic dairy products, and a practice sample all in a proof range of 33 to 92°. Each collaborator was requested to determine the proof of each sample of the 4 pairs of blind duplicate samples and report results in both specific gravity and proof units.

Alcohol in Liqueur-type and Alcoholic Dairy Products

Densitometric Method

Official First Action

(Applicable to products contg dissolved solids)

9.D01 ***Principle***

Sample is distd in special all-glass still and % alcohol in distillate is detd by density meter.

9.D02 ***Apparatus and Reagents***

(**a**) *Liqueur still.*—All Ace Glass, Inc., Cat. Nos: (*1*) *Boiling flask.*—300 mL with 35/20 joint (No. 6902-25).

(*2*) *Vigreaux distilling column.*—With 35/20 joints, 50 cm long (measured from lowest to highest indents) × 2.2 cm od (No. 36578-94).

(*3*) *Connecting adapter.*—With 35/20 joints (No. 35125-99).

(*4*) *Liebig condenser.*—With 35/20 female joint on top and straight 7 mm od tube as outlet (H_2O jacket length ca 38 cm) (No. 35994-96).

(*5*) *Condenser outlet connector tube.*—20 cm long × 7 mm od glass tube.

(*6*) *Clamps.*—3 pinch-type clamps, size 35 (No. 7670-10).

(*7*) *Volumetric flasks.*—50 mL (No. 7102-12).

(**b**) *Density meter.*—Mettler/Paar DMA 55D (Mettler Instrument Corp., Hightstown, NJ 08520).

(**c**) *Silicone antifoam.*—GE-66, GE-70 silicones, or equiv.

9.D03 ***Assembly of Distillation Equipment***

Mount Vigreaux column and condenser vertically on sep. ring stands. Apply film of silicone grease to glass joints and use pinch clamps to secure adapter to top of Vigreaux column and condenser. Connect condenser connector outlet tube to end of condenser with a 10 × 1/4 in. piece of Tygon tubing.

9.D04 ***Distillation Procedure***

Liqueurs.—Place 50 mL vol. flask contg >50 mL sample in const temp. bath. When sample temp. has stabilized, adjust vol. to mark. Quant. transfer sample to 300 mL boiling flask, rinsing vol. flask with three 8 mL portions of H_2O and adding rinsings to boiling flask. Add 1 or 2 glass beads to boiling flask and 1 or 2 drops of silicone antifoam, if sample foams. (Silicone spray *cannot* be used as antifoam because many sprays contain volatile solvs.) Connect boiling flask contg sample to bottom of Vigreaux column with pinch clamp. Add 2.5 mL H_2O to original vol. flask and use flask as distillate receiver. Submerge extension tube from condenser into H_2O in receiver set in ice bath.

Place screen on support clamp under boiling flask and heat flask with gas burner or elec. heater. Do not use elec. heating mantle because solids will carbonize on inside wall of flask. Lower receiver after ca 42 mL distillate has been collected to permit all condensate to drain from condenser into receiver. Permit last 6 mL distillate to drip into receiver to rinse condenser and extension tube. Total distn time should be >25 but <40 min. Remove 50 mL vol. flask, stopper, swirl to mix contents, and place in const temp. bath. When sample temp. has stabilized to same temp. as before distn, add H_2O to adjust vol. to mark, and mix.

Air bubbles in fresh distillates may be dissipated by placing vol. flask momentarily in ultrasonic bath, by tapping flask gently on the side, or by allowing it to stand for some period before measuring proof.

Alcoholic dairy products.—Treat as described above, except use total of 50 mL H_2O to transfer sample from vol. flask to boiling flask.

9.D05 ***Determination***

Det. % alcohol in distillate by **9.C03–9.C07.**

Results and Discussion

The collaborative proof results are shown in Table 1. For statistical evaluation, the data are grouped into sample pairs, i.e., Samples 3 and 8, 2 and 6, 5 and 7, 1 and 4. When the ranking test

Table 1. Proof determination of liqueurs and alcoholic dairy products

	Sample							
Coll.	3	8	2	6	5	7	1	4
1[a]	33.70	33.73	47.45	47.47	72.95	72.95	92.08	91.92
2	33.86	33.80	47.45	47.52	73.04	73.11	92.30	92.26
3	33.91	33.89	47.77	47.80	73.53	73.48	92.64	92.53
4	33.78	33.89	47.58	47.49	73.09	72.85	92.20	92.18
5	33.97	33.97	47.79	47.80	73.44	73.46	92.45	92.67
6	33.87	33.95	47.51	47.53	72.94	72.66	91.94	92.08
7	33.78	33.78	47.63	47.60	73.25	73.15	92.79	92.37
8	33.84	33.84	47.49	47.51	73.11	73.04	92.25	92.35
9	33.78	33.89	47.56	47.99	73.04	73.09	91.99	92.04
10	33.95	33.95	47.66	47.66	73.28	73.26	92.39	92.38
Mean	33.86	33.88	47.60	47.66	73.19	73.12	92.33	92.32
SD	0.073	0.069	0.119	0.173	0.198	0.264	0.278	0.204
CV, %	0.21	0.20	0.25	0.36	0.27	0.36	0.30	0.22

[a] Results eliminated by ranking test.
Variance ratios:

between-labs, $MS_L/MS_{LS} = 3.10$

lab–sample interaction, $MS_{LS}/MS_O = 4.28$

Repeatability SD = 0.097
Reproducibility SD = 0.198
Std error of mean = 0.19

(4) was applied, the results reported by Collaborator 1 were significantly low and were excluded from further statistical analyses.

Analysis of the acceptable data indicated that the variance ratios (MS_L/MS_{LS} (between laboratories) and MS_{LS}/MS_O (laboratory–sample interaction) were 3.10 and 4.28, respectively. The between-laboratory ratio is not significant at the 95% confidence level, while the laboratory-sample interaction value is significant. No consistent laboratory bias exists. The repeatability and reproducibility standard deviations were 0.097 and 0.198, respectively, while the standard error of mean was 0.19. The reproducibility standard deviation and standard error of mean values are similar to those reported in previous collaborative studies (3, 5) using a density meter to measure proof of ethanol–water solutions.

Advantages of using this new distillation unit are that it is inexpensive, requires a small amount of space, and is simple to operate. The 50 cm Vigreaux column provides efficient separation of ethanol from solids and water contained in the liqueur sample. Accuracy and other advantages of the density meter to measure proof have been reviewed (3).

The proof results in Table 1 are acceptable considering that the proof of ethanol in distilled alcoholic products is reported to 0.1°. They indicate the state of the art for determining the proof of alcoholic products containing high concentration of dissolved solids which traditionally are difficult to proof accurately.

Comments of Collaborators

Collaborator 6 reported that some of the contents of Sample 7 had been lost during shipment due to an inadequately torqued cap.

A 550 or 750 watt electric heater equipped with a rheostat control was used by Collaborators 5 and 7 instead of a gas burner to heat the boiling flask. One recommended heater is sold by Precision Scientific Co. (Cat. No. 61560 with 2¾ in. upper refractory, Cat. No. 61836).

Use of a heat shield between the boiling flask and distillate receiver was recommended by Collaborator 8. This modification can be avoided by using a longer glass extension tube and/or Tygon tubing to lower the distillate receiver and increase its distance from the boiling flask. With these precautions, the possibility of heat from the boiling flask affecting the contents of the distillate receiver surrounded by an ice bath is minimal.

The question was asked why this method is being evaluated for testing only liqueurs and alcoholic dairy products and not all alcoholic products containing ≥600 mg dissolved solids/100 mL. This AOAC collaborative study was initiated to develop a standard micro proofing procedure for the liqueur industry. An alcoholic

dairy product was also included because several members of the U.S. distilling industry requested a standard proofing procedure for these products. It was not possible to include all the different types of alcoholic products containing dissolved solids in this study because of the number of additional samples required. However, as noted in the introduction, this procedure is currently used by Seagram laboratories to determine the proof of liqueurs, alcoholic dairy products, whiskys, brandies, rums, and wines.

Recommendation

It is recommended that this method for determining the proof of liqueurs and alcoholic dairy products be adopted official first action.

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Gas Chromatographic Determination of Ethanol in Wine: Collaborative Study

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After a survey of laboratories using gas chromatographic procedures for wine alcohol analysis, 3 sets of columns and operating parameters were chosen on the basis of comparative reproducibility evaluations run in this laboratory. Fourteen collaborators participated in the study and were given their choice of any one of the 3 columns: (*1*) 1.8 m × 2 mm id glass, packed with 80–100 mesh Poropak QS; (*2*) 1.8 m × 2 mm id glass, packed with 0.2% Carbowax 1500 on 80–100 mesh Carbopack C; (*3*) 1.8 m × 6.35 mm copper, packed with 3% Carbowax 600 on 40–60 mesh Chromosorb T. Standards and samples were diluted 1:100 with 0.1% v/v *n*-butanol in water before analysis. Eight samples containing from 7.3 to 23.6% ethanol (v/v) were submitted as randomly coded duplicates along with a calibration standard and a practice sample. Two injections each of the diluted duplicates were made. On the basis of the reported results, 2 laboratories were rejected as outliers. The results of the remaining 12 collaborators showed an average reproducibility of 0.142 and a repeatability of 0.036. The method has been adopted official first action.

The determination of ethanol is one of the most critical analyses performed in the wine industry (1). As greater numbers of samples are analyzed, the need for a method that is specific, accurate, precise, and relatively rapid becomes more apparent. Gas chromatography would appear to meet all these requirements. The determination of alcohol in wine by gas chromatography was investigated as long ago as 1960 (2). Unfortunately, at that time, thermal conductivity detection was used and the resulting water peak caused a relatively lengthy analysis time. In addition, modern methods of integration were not available, and the ball and disk integrator that was in common use was relatively cumbersome and time-consuming. So, while the procedure was capable of the desired specificity, accuracy, and precision, it did not offer the speed or potential for automation to make it a practical technique. The introduction of the flame ionization detector and high-speed calculating integrators permitted refinement of this technique and a practical procedure was developed (3). This procedure was put into routine use in our laboratory and, after several years and many thousands of analyses, was deemed to be superior in almost all respects to other procedures available for this determination. We felt that the method should be subjected to a collaborative study with the possibility of recommending it as an official AOAC method. We also felt that workers in this field should be surveyed to determine if improvements might exist in the method we were using.

A number of potential collaborators for this study were asked if they were currently using a gas chromatographic procedure for the determination of ethanol in wine, and, if so, to give the specific operating parameters of the procedure. Of the 14 potential collaborators who responded, 9 indicated that they were indeed using gas chromatographic methods to one extent or another for this determination.

All procedures described were evaluated in this laboratory. They were compared on the basis of precision, speed of analysis, and chromatographic qualities. Three chromatographic columns and operating procedures were determined to be superior and roughly equal. It was decided that the collaborators should be given their choice of any one of 3 columns to use in this determination, because it would be likely that one of the 3 types would be readily available to participants in the study. Because the procedure involved dilution of the sample with water containing an internal standard, the normal collaborative study was modified so that the precision of the chromatography and dilution

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techniques could be evaluated separately as well as together to establish real and potential sources of error.

Collaborative Study

Fourteen collaborators participated in this study. Each collaborator was supplied with an ethanol–water calibration standard prepared in this laboratory and sealed in glass ampules. This standard was prepared from purified ethanol and water and its alcohol content was determined by averaging results from refractometer, pycnometer, hydrometer, and dichromate oxidation determinations. A stabilized wine (Standard Reference Material 1590) with a known ethanol content at 60°F (18.51% v/v) is now available from the National Bureau of Standards. This would be used as a primary standard for the official method. In addition, each participant was supplied with a practice wine sample with a known ethanol content. Participants were instructed to perform the analysis as directed with the practice sample until results indicated proficiency with the method.

Participants were instructed to refrigerate samples upon receipt to prevent refermentation before the actual analysis. Eight sets (16 total samples) of blind duplicates (a and b) of randomly coded wine samples were submitted for analysis. These ranged in ethanol content from slightly over 7% to just under 24% ethanol by volume. Each diluted sample was to be injected twice to permit evaluation of chromatographic reproducibility. Because dilution of the samples with a solution containing an internal standard is a critical portion of the analysis, respondents were asked to describe their dilution procedures as well. This factor was also to be included in the statistical analysis to see if the dilution procedure had a significant effect on the accuracy of the determination.

Ethanol in Wine
Gas-Liquid Chromatographic Method
Official First Action

11.D01 *Apparatus and Reagents*

(**a**) *Gas chromatograph.*—With flame ionization detector, integrator, heated on-column injector, and any one of the following columns and corresponding operating conditions:

(*1*) 6 ft × 2 mm id glass, packed with 80–100 mesh Poropak QS.

(*2*) 6 ft × 2 mm id glass, packed with 0.2% Carbowax 1500 on 80–100 mesh Carbopack C (Supelco Chromatography Supplies, Supelco Park, Bellefonte, PA 16823).

(*3*) 6 ft × 1/4 in. Cu, packed with 3% Carbowax 600 on 40–60 mesh Chromosorb T.

Conditions	Col. 1	Col. 2	Col. 3
Carrier gas:	N	N	N
Flow rate, mL/min:	30	15	55
Oven temp.	200°	105°	88°
Injector temp.	225°	175°	150°
Detector temp.	225°	175°	150°

Adjust air and H for flame detector to optimum for carrier gas flow of column used. Adjust electrometer sensitivity to provide ≥50,000 counts of integrator counts for internal std peak.

(**b**) *Diluter.*—Capable of ±0.1% precision.

(c) *Internal std soln.*—0.1% (v/v) *n*-butanol in H_2O.

(**d**) *Alcohol std soln.*—Prep. alcohol–H_2O soln contg approx. % alcohol expected in sample. Det. exact % alcohol by pycnometer, **9.013(a);** refractometer, **9.016;** hydrometer, **9.014;** or other appropriate AOAC method, or use Std Ref. Material 1590, Stabilized Wine (NBS).

11.D02 *Determination*

Dil. alcohol std soln 1:100 with internal std soln. Inject at least three 1.0 μL aliquots and det. av. response ratio of area of alcohol peak to area of *n*-butanol peak (*RR'*). Dil. sample 1:100 with internal std soln, inject 1.0 μL, and det. response ratio (*RR*).

$$\% \text{ alcohol} = (RR \times \% \text{ alcohol in std})/RR'$$

Results and Recommendations

All results of the study are given in Table 1. It can be seen that Collaborator 5 used a different solid support in his column, but the data did not appear inconsistent and were included in the evaluation. The ranked results in Table 2 indicate that the results of Collaborators 3 and 4 lie outside of the 5% 2-tail limits for 16 samples and 14 collaborators. Table 1 shows the calculated means and standard estimate of error with and without those 2 collaborators included. All subsequent statistical calculations given omit the data from these 2 collaborators. Although a few individual results could be excluded according to Dixon's criteria, the authors felt that no results were so far out of line that they would be atypical in a routine application of the procedure, so all data were retained.

Analysis of variance was performed on the

Table 1. Individual collaborative results for the determination of ethanol in wine by gas chromatography

Coll.	Column[a]	Dilution (A) Automatic (M) Manual	Injection	Samples: Burgundy a	Burgundy b	Sherry a	Sherry b	Apple wine a	Apple wine b	Sweet vermouth a	Sweet vermouth b	Vin rose a	Vin rose b	Fortified base wine a	Fortified base wine b	Port a	Port b	Chablis a	Chablis b
1	A	A	1st	13.34	13.38	17.52	17.47	7.45	7.40	15.94	15.97	11.47	11.51	23.53	23.55	18.56	18.57	11.22	11.30
			2nd	13.30	13.39	17.51	17.38	7.46	7.39	15.90	no data	11.39	11.48	23.54	23.55	18.53	no data	11.24	11.29
2	A	A	1st	13.26	13.23	17.79	17.60	7.37	7.22	15.99	16.01	11.42	11.26	23.77	23.79	18.78	18.70	11.17	11.15
			2nd	13.17	13.20	17.84	17.58	7.30	7.22	16.06	16.07	11.40	11.39	24.00	23.82	18.82	18.74	11.06	11.15
3[b]	A	A	1st	13.27	13.42	17.57	17.57	7.49	7.60	15.86	16.14	11.57	11.49	23.57	23.64	18.60	18.87	11.33	no data
			2nd	13.29	13.53	17.55	17.67	7.52	7.62	16.05	16.11	11.60	11.47	23.67	23.65	18.59	18.85	11.47	no data
4[b]	A	A	1st	13.08	12.74	17.23	16.99	7.12	7.16	15.55	15.86	11.01	10.31	22.95	22.90	18.20	18.16	10.87	10.90
			2nd	13.03	12.91	17.23	16.95	7.12	7.16	15.56	15.85	11.02	10.29	22.71	22.93	18.22	18.16	10.88	10.94
5	D	A	1st	13.09	13.06	17.53	17.35	7.30	7.19	15.84	15.72	11.19	11.17	23.46	23.47	18.60	18.43	11.13	11.04
			2nd	13.06	13.07	17.53	17.37	7.26	7.17	15.89	15.74	11.15	11.18	23.51	23.49	18.57	18.42	11.09	11.01
6	A	A	1st	13.15	13.16	17.51	17.51	7.31	7.31	15.96	15.88	11.25	11.29	23.72	23.68	28.58	18.57	11.14	11.10
			2nd	13.16	13.17	17.50	17.57	7.30	7.27	15.96	15.90	11.26	11.22	23.67	23.74	18.64	18.60	11.13	11.16
7	B	M	1st	13.14	13.29	17.47	17.19	7.33	no data	15.77	15.98	11.34	11.23	23.11	23.43	18.35	18.56	10.72	11.16
			2nd	13.20	13.19	17.33	17.29	7.23	no data	15.75	15.94	11.45	11.26	23.09	23.64	18.43	18.63	10.75	11.11
8	C	M	1st	12.99	12.73	17.44	17.36	7.33	7.24	15.93	15.77	11.19	11.22	23.39	23.52	18.41	18.51	11.02	10.98
			2nd	12.99	12.71	17.42	17.32	7.35	7.21	15.93	15.78	11.19	11.26	23.48	23.48	18.44	18.44	11.07	11.01
9	B	M	1st	13.04	13.20	17.58	17.50	7.39	7.45	16.16	15.72	11.58	11.55	23.09	23.33	18.56	18.61	11.47	11.45
			2nd	13.04	13.20	17.58	17.60	7.38	7.46	16.16	15.73	11.59	11.56	23.21	23.37	18.58	18.61	11.47	11.44
10	C	M	1st	13.21	13.33	17.58	17.68	7.28	7.35	16.10	16.28	11.40	11.32	24.14	23.82	18.63	18.66	11.28	11.18
			2nd	13.15	13.44	17.61	17.59	7.27	7.36	16.07	16.30	11.42	11.29	24.09	23.87	18.77	18.58	11.27	11.12
11	A	A	1st	13.19	12.95	17.55	17.46	7.24	7.18	15.87	15.81	11.23	11.25	23.66	23.62	18.55	18.45	11.04	10.99
			2nd	13.20	12.96	17.55	17.44	7.26	7.20	15.90	15.81	11.25	11.25	23.60	23.60	18.63	18.48	11.05	11.01
12	A	A	1st	13.15	12.99	17.49	17.41	7.32	7.31	15.68	15.89	11.22	11.37	23.47	23.51	18.85	18.68	11.18	11.18
			2nd	13.19	13.00	17.54	17.42	7.23	7.32	15.68	15.94	11.20	11.36	23.47	23.45	18.71	18.69	11.18	11.17
13	A	A	1st	13.17	13.12	17.55	17.49	7.31	7.31	15.98	15.86	11.33	11.31	23.43	23.51	18.57	18.51	11.10	11.13
			2nd	13.18	13.15	17.51	17.48	7.30	7.30	15.97	15.85	11.35	11.32	23.46	23.49	18.54	18.51	11.11	11.15
14	A	A	1st	13.18	13.11	17.49	17.41	7.31	7.31	15.90	15.91	11.34	11.34	23.41	23.45	18.58	18.48	11.14	11.14
			2nd	13.16	13.12	17.51	17.41	7.30	7.30	15.92	15.91	11.31	11.30	23.43	23.49	18.55	18.51	11.12	11.12
	Aggregate $\bar{x}$			13.16	13.13	17.52	17.43	7.32	7.31	15.91	15.92	11.33	11.26	23.49	23.53	18.57	18.55	11.13	11.13
	Aggregate s			0.09	0.21	0.13	0.17	0.09	0.12	0.15	0.16	0.16	0.29	0.32	0.22	0.15	0.16	0.19	0.13
	CV, %			0.69	1.60	0.72	1.00	1.30	1.70	0.98	0.98	1.40	2.60	1.40	0.94	0.82	0.86	1.70	1.20
Without colls 3 & 4	$\bar{x}$			13.14	13.13	17.54	17.45	7.32	7.29	15.93	15.90	11.33	11.32	23.53	23.57	18.59	18.56	11.13	11.15
Without colls 3 & 4	s			0.09	0.18	0.10	0.11	0.06	0.08	0.13	0.16	0.12	0.11	0.28	0.15	0.13	0.09	0.17	0.12
CV, %[c]				0.66	1.40	0.59	0.66	0.83	1.20	0.81	0.98	1.10	0.96	1.20	0.63	0.67	0.50	1.50	1.10

[a] Column A = 3% Carbowax 600 on Chromosorb T
Column B = 0.2% Carbowax 1500 on Carbopack C
Column C = Poropak QS
Column D = 3% Carbowax 600 on Chromosorb W.

[b] Excluded from statistical calculations.

[c]

No. of colls (excluding 3 & 4)	12
No. of wines analyzed in duplicate:	8
Alcohol range, %	7.3–23.6
Range of coefficients of variation, %	0.50–1.5
Average coefficient of variation, %	0.92

Table 2. Ranked results for collaborative study of ethanol in wine

	Sample								
Coll.	Burgundy	Sherry	Apple wine	Sweet vermouth	Vin rose	Fortified base wine	Port	Chablis	Total
1	13	7	12.5	10.5	12	9	8	12	84
2	11	14	5	12	11	13	14	8	88
3	14	12	14	13	13	11	12.5	13	102.5
4	2	1	1	1	1	1	1	1	9
5	3	4	3	2.5	2	7.5	4	5	31
6	8.5	10	8.5	8.5	5	12	10	8	70.5
7	10	2	5	6	7.5	3	3	2	38.5
8	1	3	5	4.5	3	5.5	2	3.5	27.5
9	6	11	12.5	10.5	14	2	9	14	79
10	12	13	11	14	10	14	11	11	96
11	4.5	8	2	4.5	4	10	6	3.5	42.5
12	4.5	5.5	8.5	2.5	6	7.5	12.5	10	57
13	8.5	9	8.5	8.5	9	5.5	6	6	61
14	7	5.5	8.5	7	7.5	4	6	8	53.5

Table 3. Analysis of variance for collaborative study of ethanol in wine

Source of variance	Sum of squares	Degrees of freedom	Mean square	Calcd variance ratio	Tabular F value (1%)
Between labs	6.848	11	0.6225 (MS_L)	25,723 (MS_L/MS_{LS})	2.25
Between samples	8691.412	15	—	—	
Lab.–sample interaction	0.004	165	2.42×10^{-5} (MS_{LS})	0.0186 (MS_{LS}/MS_0)	1.00
Between replicates	0.25	192	1.302×10^{-3} (MS_0)	—	
Total	8698.514	383			
Est. of repeatability SD 0.036					
Est. of reproducibility SD 0.142					

Table 4. Relative difference between injections and between duplicates (with injections averaged)

Item		Automatic diln	Manual diln
Injections	$\bar{x}$	0.227	0.297
	s	0.268	0.281
	n	126	63
Duplicate samples	$\bar{x}$	0.447	1.020
	s	0.441	0.785
	n	62	31

data of the 12 remaining laboratories, using the method outlined by Steiner (4). The results of these calculations are shown in Table 3. The laboratory–sample interaction variance ratio is not significant ($p > 0.10$). Since this term is a measure of the variance of the method with the effects of laboratories and samples eliminated, it shows that the variability inherent in the method is small. The large laboratory variance ratio indicates that very significant biases are possible between different laboratories. Unfortunately, the laboratory variance ratio is calculated by using the laboratory sample mean square as the denominator, which in this case is a very small number, making the resultant ratio extremely large. While it appears that there are significant differences between some laboratories (as indicated by the criterion of Thompson and Wilke), they are not of the magnitude obtained here.

One of the objectives of the study was to evaluate the precision of the chromatography and that of the dilution techniques, either automatic (mechanical) or manual, using volumetric glassware. The following formulas were applied to the data of Table 1 for this comparison:

Table 5. Average analyses for ethanol in wines by chromatographic column

Column	Wine A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	ΣXw
A	13.19	17.52	7.32	15.87	11.31	17.43	23.50	18.58	11.14	13.14	7.31	15.93	11.12	11.23	18.56	23.52	236.67
B	13.11	17.49	7.33	15.96	11.49	17.40	23.13	18.48	11.10	13.22	7.46	15.84	11.29	11.40	18.60	23.44	236.74
C	13.09	17.51	7.31	16.01	11.30	17.49	23.78	18.56	11.16	13.05	7.29	16.03	11.07	11.27	18.55	23.67	237.14
ΣXc	39.39	52.52	21.96	47.84	34.10	52.32	70.41	55.62	33.40	39.41	22.06	47.80	33.48	33.90	55.71	70.63	710.55

$n = 48$

$$\text{c.t.} = \frac{(\Sigma x)^2}{n} = 10\,518.36$$

$$\text{TSS} = \Sigma x^2 - \text{c.t.} = 1093.58$$

$$\text{Column S S} = \frac{\Sigma(\Sigma Xw)^2}{16} - \text{c.t.} = 0.0085$$

$$\text{Wine S S} = \frac{\Sigma(\Sigma Xc)^2}{3} - \text{c.t.} = 1093.20$$

Source of variance	Sum of squares	Degrees of freedom	Mean square	Variance or *F*-ratio	Tabular $F_{0.01}$
Total	1093.58	47	—	—	—
Columns	0.0085	2	0.0043	0.345	5.39
Wines	1093.20	15	72.88	5909	2.70*
Error	0.37	30	0.0123	—	—

* Significant.

Relative difference between 1st (X_1) and 2nd (X_2) injections (chromatographic precision):

$$D_{\text{rel.(inj)}} = \left| \frac{X_1 - X_2}{\frac{1}{2}(X_1 - X_2)} \right| 10^2$$

Relative difference between 1st (a) and 2nd (b) dilutions (duplicate samples):

$$D_{\text{rel.(dil)}} = \left| \frac{\frac{1}{2}(X_{a1} + X_{a2}) - \frac{1}{2}(X_{b1} + X_{b2})}{\frac{1}{2}[\frac{1}{2}(X_{a1} + X_{a2}) + \frac{1}{2}(X_{b1} + X_{b2})]} \right| 10^2$$

Differences were then segregated according to dilution method, and the means and standard errors were calculated.

The results of this evaluation are presented in Table 4. Chromatographic precision is excellent, which is obvious from a cursory inspection of the repeat injection data in Table 1. The poorer performance of manual dilution compared with mechanical dilution is evident. Previous work (3) has shown the necessity of this dilution ratio to keep in the optimum linear range of flame ionization detectors and electrometers in common use. Because one of the advantages of this procedure is its potential for automation, a mechanical dilutor would comprise a logical part of the system. There was no significant difference between injections.

The effects of columns were studied by averaging the results for wines by columns and applying 2-way analysis of variance (Table 5). Once again, there are highly significant differences between the wines. The differences between columns are not significant.

The overall advantages of this procedure in terms of its specificity, precision, and adaptability to full or partial automation make it extremely attractive to laboratories which must run large numbers of ethanol determinations. The good precision figures shown in the study can be improved even further if the dilution technique is restricted to the use of a high quality mechanical device. As for most procedures, continued experience with the method will yield better results than obtained in this study. It is recommended, therefore, that the method be adopted official first action.

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DECOMPOSITION IN FOODS

Gas Chromatographic Determination of Dimethylamine and Trimethylamine in Seafoods

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The volatile amines dimethylamine (DMA) and trimethylamine (TMA) are common degradation products of TMA-oxide in marine fish. Both compounds are used as important indicators of quality in seafoods. DMA is produced along with an equimolar quantity of formaldehyde by action of an endogenous enzyme found primarily in gadoid fish. TMA is produced in fresh, but not frozen seafoods by a bacterial enzyme. The current AOAC method for determination of TMA in fish is based on the colorimetric estimation of TMA as a picrate salt. This method is not specific for TMA; ammonia, monomethylamine, and DMA also form corresponding picrate salts. Gas chromatography provides a means of separating and quantitating the individual volatile amines. A simple perchloric acid extract of fish is neutralized with potassium hydroxide and extracted with benzene. The amines are then separated by gas chromatography on a porous polymer packed column and detected by using a nitrogen-phosphorus-specific flame ionization detector. The method provides simple, rapid simultaneous quantitation of DMA and TMA, and is applicable to a wide variety of seafoods.

Dimethylamine (DMA) and trimethylamine (TMA) are common degradation products of trimethylamine oxide (TMAO), and are often used as indicators of quality in seafood products. DMA and an equimolar amount of formaldehyde (FA) are produced by action of an endogenous enzyme on TMAO (1–4). FA produced in this reaction is thought to induce textural toughening by cross-linking myofibrillar proteins (5, 6). FA is difficult to quantitate due to irreversible binding of a portion of the FA to the proteins. For this reason, DMA content is sometimes used to infer the presence of FA. Several researchers have found that DMA content correlates well with textural degradation of gadoid fish during frozen storage and that on a molar basis, less FA is recovered than DMA (7–10). DMA itself may be important because of its role as the potential precursor of the carcinogenic compound *N*-nitrosodimethylamine (7, 11, 12). DMA is produced primarily during frozen storage but recent research has shown that under anaerobic storage conditions at temperatures above freezing, DMA can be produced in copious amounts (13).

TMA is the other volatile alkylamine of interest in seafood quality. TMA is produced in fresh, but not frozen, fish by action of a bacterial enzyme on TMAO (14).

Several methods are commonly used to quantitate DMA and TMA; each has certain advantages and disadvantages. Probably the most commonly used method for measuring DMA is the method of Dowden (15) or the modifications described by Dyer and Mounsey (16). These 2 methods are based on the colorimetric determination of DMA as a yellow copper dimethyldithiocarbamate salt. This reaction is specific for DMA.

A wider variety of methods are available for measuring TMA but they tend to be less specific. The Conway micro-diffusion method of Beatty and Gibbons (17) was among the earliest methods used for quantitating TMA in fish, but the method suffers from interference by DMA, and from a number of other problems (18). Dyer's method (14) or its modifications (19–24) are probably the best known and most widely used methods. The current AOAC official method for determination of TMA in seafoods (25) is based on Dyer's method (14). There is presently no AOAC method for determination of DMA in seafoods.

It has been shown that in species capable of

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producing substantial quantities of DMA, the TMA values obtained by using Dyer's method (14) may be in error (18, 22-24, 26). In at least 2 cases, suspect data have been published in the literature. In one paper, it was initially reported that TMA (determined by Dyer's method (19, 20)) was being produced in frozen cod (27). In a subsequent paper it was shown that it was actually DMA that was being produced (28). The following year a paper by another group was published, which described the microbiological changes in fresh cod in relation to the oxygen permeability of the packaging material (29). This paper reported TMA contents determined by Dyer's method (14), and in a recent paper it was suggested that it was primarily DMA, not TMA, that was being produced under their experimental conditions (13).

An analytical procedure for TMA determination has also been described which uses a modified ammonia-specific ion electrode. This modification renders the electrode more selective for TMA but it is subject to interference from ammonia, DMA, and other amines (30).

The interference problem associated with the TMA methods limits their applicability to only those species that do not produce significant quantities of DMA. Many researchers have turned to gas chromatography (GC) as the method of choice for quantitating volatile amines. The attraction of GC is that the amines can be physically separated to be quantitated individually. Numerous GC methods have been described, which vary in details as to sample preparation, column used, and method of detection.

Hughes (31) described an early method in which the volatile amines were extracted from fish with trichloroacetic acid (TCA). The TCA extract was neutralized and the amines were steam-distilled into hydrochloric acid. The amine hydrochlorides were freeze-dried and redissolved in water. The aqueous solution of amine hydrochlorides was injected onto a short precolumn containing dry soda lime to liberate the free amines, which were then separated on an analytical column packed with *n*-hendecanol–*n*-octadecane (4 + 1 v/w) on acid- and alkali-washed Celite. The separated amines were detected on a unique titration cell detector. This method is fairly typical of other described methods in that the amines were extracted from the fish with an acid, and recovered as a nonvolatile salt after steam distillation. Gruger (32) used perchloric acid (PCA) instead of TCA, and recovered the amines as hydrosulfate salts after steam distillation. The salts were then washed with methylene chloride and ethyl ether, and freeze-dried. The dried salts were weighed into a volumetric flask, KOH was added, and the mixture was diluted to volume with methyl octanoate. After shaking, the methyl octanoate was injected onto an analytical column packed with untreated Chromosorb 103. The separated amines were detected by a flame ionization detector (FID). Ritskes (33) extracted the amines with TCA and recovered them as hydrochloride salts after steam distillation. The amine-containing hydrochloride was neutralized and an aliquot was injected onto an alkaline precolumn. Compounds were separated on an analytical column containing 15% Carbowax 400 plus 5% polyethylenimine on Chromosorb W/NAW. The separated amines were detected by FID. Keay and Hardy (34) extracted the amines with PCA and recovered them as amine hydrochlorides after steam distillation. The hydrochloride was directly injected onto the analytical column of Dowfax 9N9 plus KOH on Silocell C-22. The first 9 cm of the column contained packing coated with a higher KOH concentration to allow in situ liberation of the free amines. The amines were detected by FID.

In an attempt to avoid the tedious steam-distillation step of these methods, Miller and coworkers used an equilibrium vapor analysis (35). A sample of minced fish was mixed with NaOH and sealed in a screw-cap vial. After heating at 60°C for 10 min, a vapor sample was removed from the headspace of the vial and analyzed by GC. The amines were separated on an analytical column packed with 2% tetraethylenepentamine on Graphon, and detected by nitrogen-phosphorus-selective FID (NPFID).

Two methods have been published which avoid some of the problems in the GC methods. Nonaka et al. (36) extracted TMA from fish by using TCA. The free amine was extracted into *n*-heptane after neutralizing the TCA with KOH. The *n*-heptane, containing the free amine, was analyzed on a column packed with 20% cetyl alcohol—2% KOH on C-22 firebrick, and detected by FID. Tokunaga et al. (37) elaborated on the above method, using it to measure both DMA and TMA. The amines were extracted from the fish by using PCA. The PCA was neutralized by KOH, and the free amines were extracted into *n*-amyl alcohol and separated on a column packed with 20% squalane–2.5% glycerine–2.5% KOH on Diasolid L; they were detected by FID. These 2 methods (36, 37) avoid some of the disadvantages of the other GC methods, and we felt

that they had good prospects for further development. In this paper, we describe our modifications to these simple extraction methods, and the suitability of our method for routine analysis of DMA and TMA in fresh and frozen seafood.

METHOD

Apparatus and Reagents

(a) *Gas chromatographic system.*—Hewlett-Packard 5840 gas chromatograph equipped with dual nitrogen-phosphorus FID (NPFID), glass-lined, heated injection ports, and Hewlett-Packard 7672 automatic liquid sampler (ALS). This microprocessor-based system allows unattended analysis of up to 50 consecutive samples at a time. 9 ft × 2 mm id glass column packed with untreated 80–100 mesh Chromosorb 103. Helium carrier gas at 20 mL/min. Hold oven isothermal at 140°C for 3.1 min, then program at 30°C/min to 190°C, and hold 2.2 min to let solvent elute from column. Detect amines by using NPFID system operated according to manufacturer's instructions. Conditions: hydrogen 3 mL/min, air 50 mL/min; collector voltage to give ca 50% maximum sensitivity to prolong life of collector assembly; detector temperature 300°C, injection port temperature 225°C. Fit injection port with disposable glass liners to prevent amine decomposition on hot metal surfaces and to facilitate cleaning injection port of alkaline residue from samples.

(b) *Hydrochloride salts of DMA, TMA, and n-propylamine (n-PA).*—Eastman Kodak.

(c) *Other chemicals*—ACS reagent quality.

(d) *Amine standard solutions.*—Prepare stock solutions from dried crystals of DMA-HCl, TMA-HCl, and *n*-PA-HCl so that each free amine is present at a final concentration of 1 mg/mL. Quantitatively transfer appropriate amount of dried amine HCl salt to 100 mL volumetric flask, add 1.0 mL HCl, and dilute to volume with water. In some cases, amines were dissolved and diluted directly in 4% (w/v) PCA. Provided no water evaporated from flasks, stock solutions were stable over several months.

Extraction of Amines from Fish

In all cases, extract amines from fish by blending 50 g muscle tissue with 100 mL 6% (w/v) PCA for 2 min at high speed in 250 mL stainless steel Waring blender jar. Filter homogenate by gravity through Whatman No. 1 paper and store in screw-cap test tube until analysis. In this extraction, it is assumed that the amines in the tissue are extracted into volume of extractant used (100 mL) plus the water contained in the tissue. Taking 80% moisture as an average for fish muscle, this amounts to 100 mL + 80% of 50 g = 140 mL. This effectively dilutes the 6% (w/v) PCA used to extract samples to 4% (w/v) PCA in the final filtrate. For samples that differ significantly from 80% moisture, exact moisture content is measured and this volume difference is used to calculate amine content.

GC Determination of DMA and TMA

Pipet 100 μL internal standard solution (*n*-PA, 1 mg/mL in water or 4% (w/v) PCA) into 15 × 125 mm screw-cap test tube.

Add 2.0 mL sample (either standard amine stock solutions or filtered $HClO_4$ extracts of tissue), 2.0 mL benzene, and 2.0 mL 65% (w/v) KOH. Cap tubes and heat in 60°C water bath for 10 min. Shake tubes 10 min on mechanical tube rotator set to give maximum mixing action.

Centrifuge tubes briefly (<500 × *g* for 15–30 s) to separate layers and sediment the potassium perchlorate precipitate. Transfer an aliquot of benzene layer to clean tube or Autosampler vial, and cap tightly. Inject 1 μL aliquot of benzene extract onto GC column.

Linearity of Amine Detection

Prepare volumetric dilutions of standard solutions of DMA, TMA, and *n*-PA in range 2.0 to 1000 μg/mL for the free amine, corresponding to amine contents of 0.17 to 87.00 mg amine nitrogen/100 g tissue, which approximates the range of values found in very fresh though poor quality fish. Analyze these dilutions by GC as described above and prepare linearity plots for each compound of interest.

Recovery of DMA and TMA from Fish

Previously frozen minced red hake was extracted with 4% (w/v) PCA or with 4% (w/v) PCA containing various levels of DMA or TMA. Two different batches of frozen red hake were used in the recovery experiments. This reflects the difference in the DMA and TMA contents of the unspiked samples in recovery experiments with *n*-amyl alcohol (Table 1) and benzene (Table 2). The extracts were analyzed as described above to determine recovery values. *n*-PA was used as the internal standard.

Results and Discussion

Figure 1 shows a chromatogram of a standard mixture of DMA, TMA, and *n*-PA, each at a concentration of 50 μg/mL. The use of the 9 ft × 2

Table 1. Recovery of DMA and TMA from minced red hake, using *n*-amyl alcohol as solvent (mg N/100 g fish)

DMA			TMA		
Added	Found	% Recovery	Added	Found	% Recovery
0	12.8		0	1.0	
	13.1			1.4	
	13.4			1.3	
	13.1 ± 0.3[a]	—		1.2 ± 0.2	—
3.1	14.8		2.4	3.8	
	15.1			3.7	
	15.0			3.7	
	15.0 ± 0.2	61.3		3.7 ± 0.1	104.2
6.2	16.9		4.7	6.3	
	16.6			6.3	
	17.9			6.3	
	17.1 ± 0.7	64.5		6.3 ± 0.0	108.5
15.5	22.5		11.9	12.8	
	21.6			12.6	
	23.0			12.9	
	22.4 ± 0.7	60.0		12.8 ± 0.2	97.5
31.1	31.6		23.7	22.8	
	34.1			24.1	
	33.7			23.8	
	33.1 ± 1.3	64.3		23.6 ± 0.7	94.5

[a] Mean ± standard deviation.

Table 2. Recovery of DMA and TMA from minced red hake, using benzene as solvent (mg N/100 g fish)

DMA			TMA		
Added	Found	% Recovery	Added	Found	% Recovery
0	5.5		0	0.1	
	6.1			0.1	
	5.4			0.2	
	5.8			0.2	
	5.4			0.2	
	5.6 ± 0.3[a]	—		0.2 ± 0.1	—
3.1	8.8		2.4	2.5	
	8.9			2.5	
	9.0			2.5	
	8.9			2.5	
	9.3			2.5	
	9.0 ± 0.2	109.7		2.5 ± 0.2	95.8
6.2	11.2		4.7	4.8	
	11.5			4.8	
	12.5			4.8	
	12.8			4.8	
	12.4			4.8	
	12.1 ± 0.7	104.8		4.8 ± 0.0	97.9
15.5	21.8		11.9	11.8	
	20.9			11.6	
	21.8			11.8	
	22.1			11.7	
	22.5			11.8	
	21.8 ± 0.6	104.5		11.7 ± 0.1	96.6
31.1	34.5		23.7	21.8	
	34.2			21.5	
	36.6			22.5	
	38.0			23.2	
	39.7			23.2	
	36.6 ± 2.3	99.7		22.4 ± 0.8	93.7

[a] Mean ± standard deviation.

mm id glass column packed with untreated Chromosorb 103 allowed almost complete separation of DMA and TMA. The internal standard, *n*-PA elutes from the column somewhat later, just before the solvent (benzene). Some slight tailing of the DMA peak is observed, but the TMA and *n*-PA peaks are fairly symmetrical. Since the benzene contains no nitrogen or phosphorus, the detector response is very small. Using a new collector element in the detector results in absolutely no response for benzene. Slight aging effects were noted for the collector, with a negative peak gradually progressing to a large positive peak as the alkaline rubidium sulfate bead in the collector is volatilized over a period of time. Benzene, however, elutes far enough away from *n*-PA that quantitation is not affected, even using old collectors which give a large positive response to benzene.

The column packing chosen for this method, Chromosorb 103, is a cross-linked polystyrene material that can provide a fairly good separation of DMA and TMA (32, 38). We felt that the porous polymer packing was superior to any packing coated with a liquid phase for the following reasons: The porous polymer packing has no liquid phase that could be lost through gradual volatilization, causing an eventual loss of column efficiency and subsequent detector contamination. The stability of Chromosorb 103 is very good. We successfully ran about 25 000 samples through one column over a 24 month period with no detectable change in performance. On the Chromosorb 103 column, the amines elute before the solvent so analysis time is limited by how fast the solvent elutes. DMA retention time is 2.34 min; for TMA, 2.75 min; and *n*-PA, 4.91 min. The solvent, benzene, begins to elute at about 6.5 min and elution is complete by about 8.0 min. The temperature program used (140°C for 3.1 min, then 30°/min to a final temperature of 190°C which is held for a further 2.2 min) takes 7.0 min to complete. The microprocessor adds about 6 min to each analysis cycle in oven cooldown and column temperature equilibrium time to yield a total analysis cycle of about 13 min. About 110 samples can be analyzed in a 24 h period under these conditions using the automatic liquid sampler for unattended operation of the GC system. About 36 samples can be injected manually in an 8 h

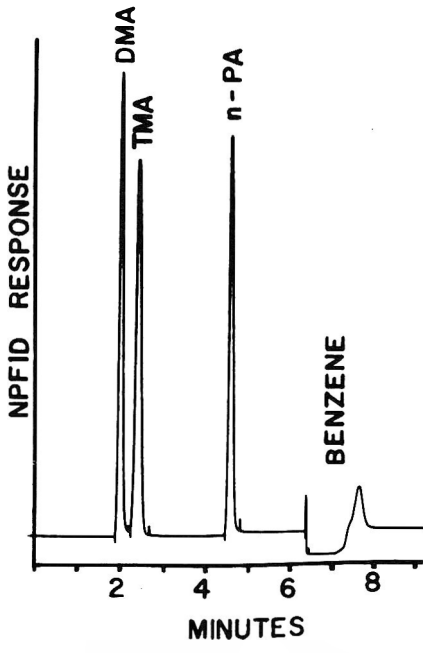

Figure 1. Gas chromatogram showing separation of dimethylamine (DMA), trimethylamine (TMA), and *n*-propylamine (*n*-PA) on 9 ft × 2-mm id glass column packed with untreated Chromosorb 103.

working day if an automatic sampler is not available.

The identity of the DMA and TMA peaks from fish extracts is presumptive, based solely on retention times. Other researchers have compared volatile amine contents obtained using both colorimetric and GC methods and found general agreement (12, 36, 39, 40). Gruger, using a Chromosorb 103-packed column, found good agreement between amines identified in tissue by GC and identifications based on thin layer chromatography–spectrophotofluorometry of corresponding 5-dimethylamine-1-naphthalene sulfonamides (32). The agreement of retention times for amines isolated from tissues and for standard amine solutions on many different packings (31–37) lends further support to the contention that the peaks in question are in fact DMA and TMA.

Figure 2 shows the linearity of the NPFID response to DMA, TMA, and *n*-PA. Each data point represents the mean of triplicates. The range of 2–1000 μg/mL approximates the range of DMA and TMA which could be separated from very fresh or very old fish. This corresponds to levels of approximately 0.2–87.0 mg DMA-N/100 g (0.01–6.21 mmoles DMA/100 g)

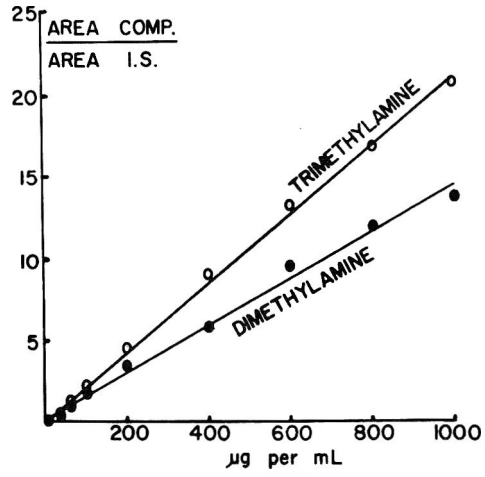

Figure 2. Linearity curves for dimethylamine and trimethylamine.

and 0.1–66.4 mg TMA-N/100 g (0.01–4.74 mmoles TMA/100 g) for TMA. Linearity curves were plotted as the ratio of the component peak area to the internal standard peak area as a function of concentration to eliminate the influence of injection volume and detector sensitivity on absolute area measurements. Correlation coefficients for the regression lines (DMA = 0.993, TMA = 0.999) were highly significant ($P \leq 0.001$).

The recovery of DMA and TMA was a problem using the *n*-amyl alcohol solvent suggested by Tokunaga et al. (37). They found recoveries of 98.0% for DMA and 96.7% for TMA at a level of 15 mg amine N/100 g for each compound. Our recoveries of DMA and TMA at various levels in minced red hake were lower and more erratic. Table 1 shows recoveries using *n*-amyl alcohol as the organic solvent. At all 4 levels, TMA recovery is good (average 101.2%) and consistent but DMA recovery was low (average 62.5%).

We also did some limited testing of other potential solvents. Substituting *n*-heptane, we obtained average recoveries of 104.3% for DMA but only 88.9% for TMA. Substituting toluene resulted in average recoveries of 88.0% for DMA and 92.6% for TMA. Using hexane, recoveries averaged 96.4% for DMA and 71.7% for TMA. Benzene gave the best overall average recovery of DMA and TMA (Table 2), 104.7 and 96.0%, respectively.

Comparisons of colorimetric and GC methods for quantitating DMA and TMA have shown that the methods for DMA give comparable values, while GC is generally superior for quantitating

TMA (12, 36, 39, 40). The method described in this paper has been used for the past 4 years for routine quantitation of DMA and/or TMA in a wide variety of seafoods. We have encountered no problems in analyzing extracts of red hake (*Urophycis chuss*), cod (*Gadus morhua*), haddock (*Mellanogrammus aeglefinus*), cusk (*Brosme brosme*), whiting (*Merluccius bilinearis*), pollock (*Pollachius virens*), white hake (*Urophycis tenuis*), American plaice (*Hippoglossoides platessoides*), blackback flounder (*Pseudopleuronectes americanus*), and squid (*Illex illecebrosus*). The method is easy to perform, fairly rapid, uses small quantities of reagents, and requires no special equipment other than the gas chromatograph. Simultaneous quantitation of both DMA and TMA is also a distinct advantage.

Recommendation

The Associate Referee recommends that this method be collaboratively studied to determine accuracy and precision, and further recommends that the current AOAC method for determination of TMA in fish (**18.031-18.033**) be compared with the GC method during the collaborative study to determine whether the present method should be retained, modified, or eliminated.

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FISH AND OTHER MARINE PRODUCTS

Identification of Trout and Salmon Bloods by Simple Immunological Technique and by Electrofocusing Patterns of Red Cell Enzyme Superoxide Dismutase

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Species identification of animal bloods is readily achieved by immunological tests. Differentiation among fish species on this basis is more difficult although considerable success has been achieved on the basis of both inter- and intra-specific differences in their serum proteins. This report describes a method for the identification of the different species of fish within the Salmonidae family and some coarse fish families on the basis of an immunological test and electrofocusing patterns of the enzyme superoxide dismutase from the red cell. The immunological technique relies on the development of a specific anti-trout (Salmonidae) serum which is used initially to differentiate the blood of a Salmonidae from other freshwater fish. Further discrimination, within the Salmonidae, is made on the basis of the different polymorphic forms of the enzyme superoxide dismutase separated in a pH 2.5 to 8 gradient. Using this technique, it is possible to differentiate among salmon, sea/brown trout, char, cheetah trout, and a number of varieties of rainbow trout.

The features used to differentiate and identify species of fish are mainly external characteristics such as color, position of fins, number of fin rays, and the number of scales in a particular "scale-row." Internal structures can also be used for particular families, e.g., vertebrae counts, pharyngeal and vomer bones, and gills. Unfortunately, many of these characteristics can be influenced and modified by the environment; therefore, a number of biochemical techniques have been developed to identify characteristics that are genetically determined and not influenced by the environment.

The forensic scientist, however, may have very little evidence for the positive identification of the fish when the available material is in the form of epidermal scales and/or blood. In the past, forensic and government chemists have relied on identification of fish scales to provide evidence in poaching cases and other illegal fishing activities.

Scales can be divided into 2 groups: cycloid and ctenoid. Cycloid scales are so-called because their exposed margin is totally smooth; this scale type is found on the majority of scale-bearing fish including the salmon (Salmonidae) and carp (Cyprinidae) families. Ctenoid scales differ in that their exposed margins carry small teeth-like structures or ctenii. Examples of fish in this group include the perch (Percidae) and pike (Esocidae) families.

Although scale morphology can be helpful in differentiating orders (1), the technique has been used to identify a number of salmon species in the canning industry (2). Such instances, however, are exceptional and more reliance has been placed in recent years on various electrophoretic techniques.

In this report, we examine the possibility of differentiating among species of fish, in particular those in the Salmonidae family, on the basis of an immunological test and from the electrofocusing patterns of superoxide dismutase (SOD) from various tissues and red cell lysates.

Electrophoretic Methods and Fish Identification

Sea Fish.—The identification of sea fish proteins by using various electrophoretic methods has been studied extensively (3–6). A valuable commercial application was devised by Thompson (4) who used a starch gel electrophoretic technique to differentiate the proteins in fillets made from 7 species of fish including perch (*Sebastes marinius*), flounder (*Platichthys flesus*), cod (*Gadus morhua*), haddock (*G. aeglefinus*), whiting (*Merluccius bilinearis*), pollock (*Pollachius virens*), and halibut (*Hippoglossus hippoglossus*). The technique has been used to expose a case of substitution in Kansas where a merchant advertized catfish but was selling pollock (4). However,

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one of the most successful methods of differentiation was based on cellulose acetate electrophoretic separation of the water-soluble proteins from fish muscle. Using this method, Lane et al. (5) distinguished among cod, haddock, pollock, and whiting. In the same year, Hill et al. (7), used an agar gel method to identify 13 species of North Atlantic fish from muscle protein in fresh, frozen, and freeze-dried samples.

Most electrophoretic methods are subject to one or more limitations that can lessen their effectiveness as routine species identification tests. In conventional electrophoresis, protein pattern variations can be caused by variations in the composition of the stabilizing media, sample application, applied voltage, current, or even the analyst's skill. In an attempt to overcome some of these problems, Lundstrom and Roderick (6) applied an electrofocusing technique to the separation of the sarcoplasmic proteins from a number of fish species including cod, haddock, whiting, and mackerel (*Scomber scombrus*). This resulted in a method that was quickly and easily performed, with protein patterns that were specific and reproducible for each species.

Freshwater Fish.—The concept of using polymorphic enzyme systems for identifying freshwater fish has been examined for a number of years. Brassington and Ferguson (8, 9) reported that the starch gel electrophoretic patterns of lactate dehydrogenase and esterases from white muscle could be used to differentiate roach (*Rutilus rutilus*), rudd (*Scardinius erythrophthalmus*), bream (*Abramis brama*), and some of their hybrids. The same electrophoretic technique was also applied by Reinitz (10) to distinguish rainbow trout (*Salmo gairdneri*) and cutthroat trout (*S. clarki*) on the basis of the enzyme phosphohexose isomerase from muscle tissue.

Although enzyme polymorphism can be demonstrated among different species of fish, Taggart et al. (11), working with various tissues from brown trout populations in both England and Ireland demonstrated a number of polymorphic enzyme systems. Of 60 different loci examined, they found that at least 22% showed polymorphism that could be useful as markers in detailed population studies. Superoxide dismutase was one of the enzymes that showed no polymorphic forms and that was readily detected by polyacrylamide electrophoresis. This enzyme has already been used to differentiate species of deer found in the United Kingdom (12). Therefore we decided to evaluate this system for differentiating species of fish, in particular the Salmonidae.

Superoxide Dismutase – Biochemical and Genetic Aspects

Superoxide dismutase (SOD) has been detected in a wide range of living things where it catalyses the dismutation of superoxide radicals to yield hydrogen peroxide, a reaction which proceeds spontaneously at pH 7.7.

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

It has been proposed that the physiological function of the enzyme is to protect organisms metabolizing oxygen against a potentially deleterious effect of superoxide free radical (13).

Polymorphism in Humans

Beckman et al. (14) showed that human superoxide dismutase is controlled by 2 separated loci SOD_A and SOD_B. SOD_B is located in the mitochondrial fractions of most tissues and organs although it is absent in red blood cells (14), and is the slowest moving of the 2 isoenzymes, with no genetic variants. SOD_A is found in all tissue except polymorphonuclear lymphocytes (14); it has a molecular weight of 33 000 and contains both Zn^{2+} and Cu^{2+} (15). SOD_A exhibits polymorphism, and 3 electrophoretic variants have been recognized, SOD_A1, 2-1, and 2. Although the SOD_A1 variant is extremely common, the 2-1 and 2 phenotypes are very rare and only occur with relatively higher frequencies in isolated communities in Northern Finland, North Sweden (16), and on the Westray Islands in the Orkneys (17).

Polymorphism in Fish

There is still some debate as to the number of loci determining superoxide dismutase in fish. Utter (18) claimed that it was a dimeric enzyme determined by a single locus in many members of the Salmonidae family. This was confirmed by Allendorf et al. (19) who reported a single locus determining one variant allele in Swedish brown trout. However, more recent studies by Taggart et al. (11) working with various tissues from Irish brown trout found evidence which suggested that, as for humans, 2 loci were involved. (Note: Since there is still some confusion over the number of loci involved, superoxide dismutase will be referred to as SOD only.)

Materials

Collection of Lysates

Whole blood was obtained from the caudal vein of freshly killed coarse fish. This blood was

spun down and washed twice with 0.85% saline solution, using a Burkhard microcentrifuge type 320. Lysates were obtained by freezing at −20°C for 30 min and then thawing at room temperature.

Tissue Extracts

Samples of liver and roe were obtained from freshly killed Carabou rainbow trout. Each sample was frozen briefly, thawed, and then homogenized in a Dounce homogenizer for 5 min in an equal volume of water (1 g/mL). The extract was then spun in an MSE high speed centrifuge at 3000 rpm for 10 min at 4°C in a centrifuge head No. 59583. The supernatant was used neat for all electrofocusing studies. In addition, some scales were removed from the upper part of a freshly killed grayling (*Thymallus thymallus*), and the basal tissue was examined for SOD activity. (Grayling was chosen for this purpose because it is readily available and has scales that are relatively large and easily removed.)

Fish Blood

The following organizations kindly donated fish bloods from their stocks, which enabled us to undertake this project.

Wessex Water Authority, Poole, Dorset: samples of all the fish listed above with the exception of the brook and cheetah trouts.

North Wales and South Western Water Authorities: a number of sea trout from their respective areas.

Freshwater Biological Association Laboratories, Ambleside, Cumbria: a large number of brown trout.

Avington Trout Farm, Arlesford, Hampshire: rainbow trout and all samples of the hybrid cheetah trout (brook trout × rainbow trout).

Berkshire Trout Farm, Hungerford: rainbow trout (Shaster strain), brown trout, and one of the two samples of American brook trout (char) (*Salvelinus alphinus*). The brown trout (*Salmo trutta fario*) held at this farm have been in existence since the early 1900s; the rainbow trout were introduced from the United States during the 1930s.

Avon Springs Trout Farm, Pewsey, Wiltshire: samples of rainbow trout blood from the American strains Carabou, Washington, Winthrope, Grampian, and White Brook. We purchased additional specimens of rainbow trout of unknown strains at 2 commercial fish farms in the Berkshire area, which sell fish to the public for consumption.

D. Pascao, University of Wales Institute of Science and Technology, Cardiff: an additional sample of brook trout blood, which was obtained in the stain form.

Details on the number of fish species examined in this study are also shown in Table 1.

Preparation of Antisera

Fish antisera.—Fifty common carp (*Cyprinus carpio*) of comparable weight, age, and condition were divided into 5 groups of 10. Each fish was infused with 0.25 mL trout serum in Freund's complete adjuvant into the interperitoneal cavity. The fish were bled twice over a 2-week period after one month, and the titre for each serum sample was determined by using the crossover electrophoretic method of Culliford (20). Aliquots (250 mL) of serum were then freeze-dried and stored at 4°C before use.

Freeze-dried trout antiserum, raised in carp, was obtained from the Huntingdon Research Laboratory under a Home Office contract. The antiserum was reconstituted in either 250 or 100 μL water.

Human antiserum.—Human antiserum was obtained from Wellcome Reagents Ltd, Beckenham, and was raised in rabbits.

Control Sera

Salmon, trout, carp, and human control sera were obtained from the Huntingdon Research Laboratory freeze-dried and were reconstituted in 0.25 mL water before use. In addition, serum samples were obtained from freshly killed brown and rainbow trout, grayling, and several members of the carp family including roach, dace (*Leuciscus leuciscus*), chub (*L. cephalus*), common carp, mirror carp, crucian carp (*Carassius carassius*), and bream. These sera were used as additional controls to test the specificity of the trout antisera.

Immunoelectrophoresis

Immunoelectrophoresis is a 2-stage process carried out in a 1% buffered agar gel cast on a 7.5 × 5 cm glass slide, using the method of Hirschfeld (21). The initial stage involves electrophoresis of the serum proteins in one direction along the length of the gel (Figure 1). When electrophoresis is complete the antiserum is placed in the narrow rectangular trough cut in the gel adjacent to the separated proteins. The gel is then incubated in a humidity chamber at room temperature (18–20°C) for 24–36 h during which time the antiserum will have diffused into the gel towards the electrophoretically separated

Table 1. Results of immunological study to evaluate specificity of anti-trout serum against serum from a number of British freshwater fish, using the Ouchterlony technique (ref. 22)

Order	Family	Species	No. examined	Immunological reaction, 1:400
Petromyzoniformes	Petromyzonidae	river lamprey (Lampetra fluviatilis)	1	–
	Clupeidae	Allis shad (Alosa alosa)	1	–
		Twaite shad (Alosa fallax)	1	–
		salmon	8	+
		brown trout	12	+
		sea trout	14	+
		American brook trout (char)	2	+
		Rainbow trout strains:		
		Carabou	4	+
Isospondyli[a]	Salmonidae	Washington	4	+
		Winthrope	4	+
		White Brook	4	+
		Grampian	4	+
		Hybrid trout:		
		cheetah trout (rainbow × brook trout)	1	+
	Thymalidae	grayling	3	+
	Osmeridae	smelt (Osmerus eperlanus)	2	–
	Cyprinidae	common carp	4	–
		mirror carp	4	–
		crucian carp	5	–
		roach	2	–
		rudd	2	–
		dace	2	–
		chub	4	–
		bream	2	–
		goldfish (Carassius auratus)	1	–
		gudgeon (Gobio gobio)	1	–
		tench (Tinca tinca)	2	–
		minnow (Phoxinus phoxinus)	1	–
	Corbitidae	spined loach (Cobitis taenia)	1	–
Haplomi	Esocidae	pike (Esox lucius)	3	–
Apodes	Anguillidae	eel (Anguilla anguilla)	1	–
Thoracostei	Gasterosteidae	three-spined stickle-back (Gasterosteus aculeatis)	1	–
Pereomorphi	Percidae	perch (Perca fluviatilis)	1	–
Scleropharei	Cottidae	bullhead (Cottus gobio)	1	–

[a] No vendace (order Isospondyli, family Coregonidae) was available.

components. As soon as the antigen and the relevant antibody meet, an opaque arc-shaped precipitate appears. The gel is then dried for 4 h in a 56°C incubator under a damp filter paper, and then stained with 0.1% Amido black solution for 15 min. Excess stain is removed by washing in methanol–water–acetic acid (1 + 1 + 0.2).

The antiserum was evaluated by using the crossover electrophoretic method of Culliford (20) and the Ouchterlony double diffusion plate system (22) in agar gels prepared in a pH 8.6 Hirschfeld buffer. Immunological studies were conducted at a 10-fold dilution of the antiserum from neat, 1:10, 1:100, and then a doubling dilution from 1:200 to 1:400 followed by a 1:2.5 dilution to 1:500, using antisera reconstituted in either 250 or 100 μL water. For the crossover studies, 3 μL serum or antiserum per well was used. For the Ouchterlony system, 8 μL serum or antiserum was used in wells cut with a Feinberg gel cutter.

The Feinberg gel cutter is designed to cut 6 wells each 3 mm in diameter, 2.5 cm apart, in a circle around a central well that is also 3 mm in diameter. The antiserum is placed in the central well and allowed to diffuse to the encircling wells which contain varying dilutions of antigen (serum).

Isoelectric Focusing Method

Apparatus

All isoelectric focusing was done on LKB 2117 Multiphor apparatus (LKB Instruments, Hicksville, NY) in conjunction with a Grant cooling system.

Construction of Glass Mold

A sandwich of 3 glass plates, two with dimensions 26 × 15 × 0.3 cm and one with dimensions 26 × 15 × 0.1 cm, was assembled, separated by a 1 mm rubber gasket located along the

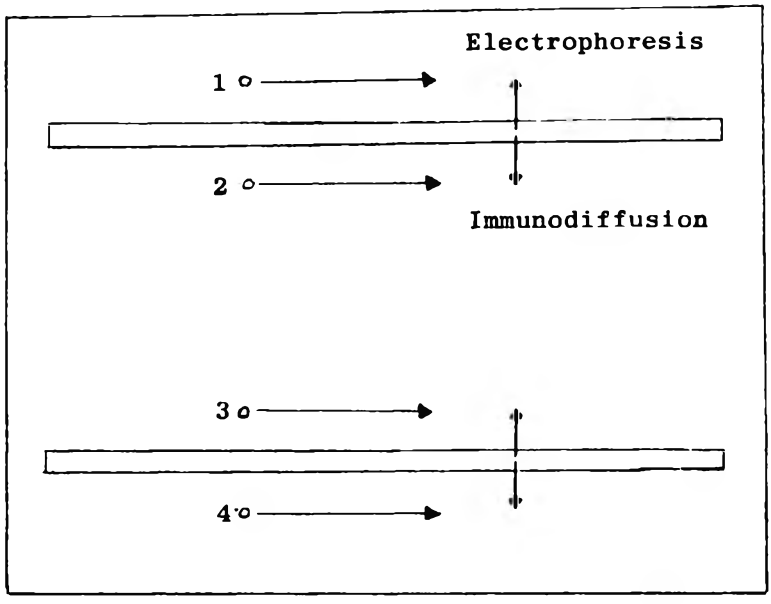

Figure 1. Diagram of immunoelectrophoretic system for evaluating antigenic profile of anti-trout serum.

outside edge. The gasket was cut at one point to allow addition of the acrylamide mixture.

Polyacrylamide Gel Preparation

(1) Stock acrylamide solution: Acrylogel (acrylamide monomer containing 5% w/w *N,N'*-methylenebisacrylamide, BDH Chemicals, Poole, UK), 66 g/L.

(2) Ingredients for one gel: Acrylogel solution, 33 mL; sucrose, 3.6 g; and 0.4 mL each of Ampholine (LKB) pH 2.5–4, pH 4–6, and pH 5–8.

The mixture was degassed for 1–2 min in a 500 mL Buchner flask by using a high vacuum pump. After the air bubbles had been removed, 0.2 mL riboflavin (10 mg%) was added. The solution was carefully introduced into the glass mold by using a 50 mL pipet. The rubber gasket was then closed and the gel was allowed to photopolymerize for 3 h under ultraviolet light.

Once polymerization was complete, the clips were removed and the sandwich of plates was placed in a refrigerator at 4°C for 15 min. The rubber gasket was then removed and the glass sandwich was carefully prised apart with a metal spatula. Cooling at 4°C for 15 min permits easier separation of the plate.

Electrode Solutions

1M phosphoric acid and 0.5% pH 5–8 Ampholine (LKB) were used as electrode solutions for the anode and cathode, respectively. The solutions were applied to the gel soaked on Whatman paper wicks (grade 17 Chroma, 1 cm wide and 1 mm thick).

Sample Application

Lysates and tissue extracts were applied to the gel by using Whatman No. 1 filter paper strips (1 × 0.5 cm). These strips were blotted before being applied to the gel approximately 3 cm from the anodic edge to prevent any residual activity left by the paper strips masking the area in the gel in which the isoenzymes were to focus.

Analytical Conditions

Initially the gels were prefocused for 30 min at 300 V and a maximum of 2 watts. The samples were applied and the voltage was maintained at 500 V until the hemoglobin entered the gel (approximately 40 min). The voltage and power settings were then increased and retained at 1200 V and 7 watts for a further 3 h. All electrofocusing experiments were conducted at 6°C.

Enzyme Visualization

Wicks and sample applicators were first removed and a frame of glass rods was made around the gel. A mixture for developing one gel was prepared by combining 0.3M Tris/HCl buffer, 33 mL; MTT tetrazolium, 5 mg; phenazine methosulfate, 5 mg; and 2% agar (cooled to 60°C), 20 g.

The combined agar-reaction mixture was

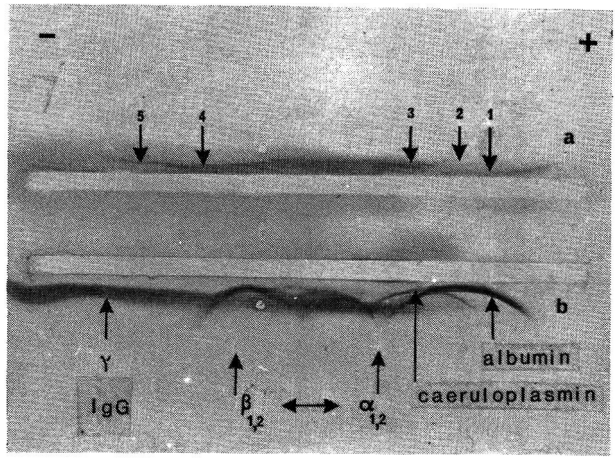

Figure 2. Immunoelectrophoretic patterns obtained for (A) neat trout serum run against anti-trout serum reconstituted in 100 μL water; (B) spectrum of precipitin arcs obtained for human serum run against a whole rabbit antiserum directed against human serum.

poured over the acrylamide gel and allowed to set. The glass rods were removed and the plate was incubated in the dark at 37°C for 1 h followed by approximately 10 min under a fluorescent strip lamp. The enzyme appeared as light patches on a dark purple background.

Since it is not possible to determine the pH profile of a gradient or the pI value of an isoenzyme once the reaction mixture has been applied, it will be necessary to carry out any pH determinations on a focused gel which has not been treated in this way.

Determination of pH Gradient

An electrofocusing gel was prepared in the manner already described and then focused for 3¾ h to ensure that the same pH gradient existed as that obtained after a normal electrofocusing run. The electrode wicks were carefully removed and a sheet of graph paper, graduated at 1 cm, 5 mm, and 1 mm intervals, was placed between the gel and the cooling plate (6°C).

A surface electrode (Pye Unicam Ingold E7) was placed in a clamp and gently lowered onto the anodic surface of the gel in a position adjacent to the first 1 cm mark. The electrode was allowed to rest under its own weight on the gel surface, and pH values were taken at 2 cm intervals along the length of the gel. This was repeated for the recurring 1 cm positions until the cathodic end of the gel was reached. The mean pH value along each centimeter division was then plotted vs gel width. This process was repeated for a further 5 gels that had been run under identical conditions. A pH gradient was then drawn on the basis of the pH values obtained from all 6 gels and was used in all subsequent pI determinations.

pI Measurements

As soon as the isoenzyme patterns were visible, the gel was carefully transferred to a piece of graph paper, identical to that used in pH gradient determinations, and was carefully aligned so that the anodic and cathodic edges of the gel were each on a 1 cm or 0.5 cm mark.

The position of the isoenzyme was then marked on the graph paper and the position was correlated with the pH gradient already determined. The individual pI values of the different isoenzymes were obtained by reading from the pH scale at the point on the gradient where the isoenzymes were found to focus.

Results and Discussion

Immunoelectrophoretic Studies

Figure 2 shows the immunoelectrophoretic patterns obtained for neat trout serum against anti-trout serum reconstituted in 100 μL water and for human serum against a whole human antiserum raised in rabbits. Comparing the patterns shows that positions 1 and 3 in Figure 2a are analogous to human albumin and caeruloplasmin while position 2 is possibly equivalent to human antitrypsin. Finally, positions 4 and 5 may represent the equivalent in fish of the IgG complex of proteins. There were indications that anti-trout serum was also reacting with the α/β range of proteins although no clearly defined arcs were ever observed. The results that we obtained when the trout antiserum was re-

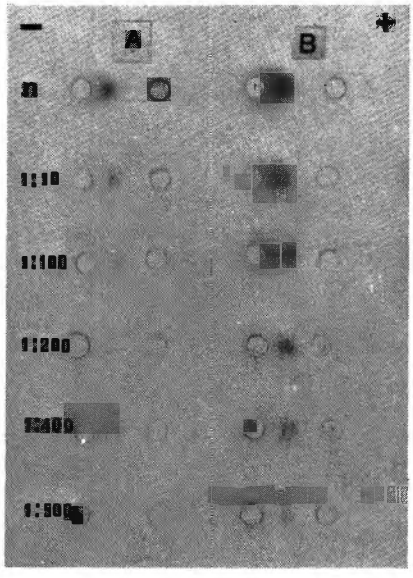

Figure 3. Precipitin reactions obtained by using crossover electrophoretic technique between anti-trout serum reconstituted in 250 (A) or 100 (B) μL water against varying dilutions of trout serum.

constituted in 250 μL water were essentially the same except that the precipitin arcs were very much weaker, especially those within the γ-globulin region.

The results of these immunological studies indicate that the anti-trout serum is not only heterogeneous but is weak when compared with the human equivalent. Furthermore, it is necessary to make a serial dilution of a bloodstain extract in order to cope with the range of component sensitivity that is likely to occur.

As a further check on the specificity of the reaction, carp serum was run against varying dilutions of common carp and trout serum using the crossover technique. The results showed that no precipitin lines appeared, which suggested that protein interaction between different species of fish was unlikely.

Crossover and Ouchterlony Studies

Figures 3 and 4 compare the crossover and Ouchterlony results obtained with brown trout serum, ranging in dilution from neat to 1:500, when run against antisera reconstituted in either 250 μL (A) or 100 μL (B) water.

With the crossover technique, using the more dilute antiserum, the results were somewhat variable in that clear precipitin lines could not always be detected with serum dilutions down to 1:200. This observation was further reinforced with the Ouchterlony method in which no clearly defined arcs were recorded much below 1:100.

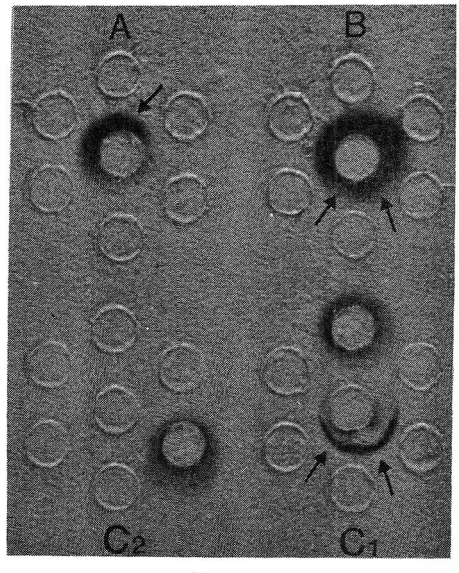

Figure 4. Ouchterlony results obtained for anti-trout serum reconstituted in either 250 (A) or 100 (B) μL water and run against varying dilutions of trout serum.

Position C_1 shows the immunological response obtained with the equivalent dilutions of human serum run against anti-human serum. Positions $C_2$1, 2, 4, 5, and 6 contain varying dilutions of anti-trout serum; position $C_2$3 contains neat human serum. All C_2 positions were run against gel buffer in center well.

When the antiserum was reconstituted in 100 μL water, the results were more encouraging. We detected a clear precipitin reaction at dilutions of 1:200 to 1:400 using either the crossover or Ouchterlony techniques. Positions C_1 and C_2 (Figure 4) represent a series of control experiments on the same plate. Position C_1 shows the series of immunological reactions obtained when neat human serum was run against antihuman (albumin) serum. Precipitin arcs were recorded between serum dilutions of 1:100 and 1:400. Positions $C_2$1, 2, 4, 5, and 6 contain varying dilutions of trout serum from neat, 1:10 down to 1:400 run against gel buffer only; position $C_2$3 contains neat human serum. No positive immunological reactions were observed in any wells; the slight staining obtained in position $C_2$3 possibly represents some precipitation into the agar gel of lipoprotein material associated with the neat human serum.

On the basis of these observations, we decided to test the antiserum in the more concentrated form against various fish species. The results are shown in Table 1. The only positive reactions were with the various Salmonids, and the grayling (family Thymallidae). No positive reaction was obtained with any of the remaining families within the order Isospondyli, namely, Clupeidae and Osmeridae. No examples of vendace (*Coregonus albula*), family Coregonidae, were available. A single positive reaction was recorded with one of the 5 crucian carp (family Cyrinidae) using the crossover technique. This result was not confirmed by the Ouchterlony method. As yet we have been unable to provide any explanation of this cross reaction although it is conceivable that serum protein allotypic variation could exist within the carp family, whose members crossbreed very readily (23).

We conclude, therefore, that this antiserum is highly specific for the members of the Salmonidae and Thymallidae families. The antiserum is best reconstituted in 100 μL water and, to obtain the best possible antibody-antigen response, a serial dilution of the extract should be made down to at least 1:200 to 1:400.

Electrofocusing Study

pH Gradient.—Figure 5 shows the profile of the pH gradient used in this study. Linearity was observed within a pH range of approximately 4.25 to 5.25.

Superoxide Dismutase.—All fish examined in this study showed SOD activity in their red cells, which could be observed in a pH range of this gradient. The intensity of the electrofocusing

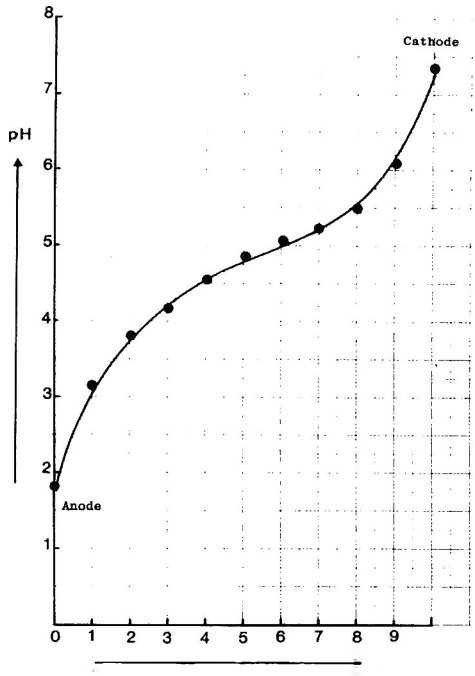

Figure 5. pH gradient obtained for pH 2.5-8 Ampholine mixture using 1% H_3PO_4 for anode and 0.5% v/v pH 5-8 Ampholine for cathode.

bands varied with each individual species although each pattern was characterized by at least one intense band, for example, as seen in the sea trout and salmon. These simple patterns contrasted quite strongly with certain members of the carp family, notably, the Chinese grass carp and mirror carp which had 3 isoenzymes.

The electrofocusing patterns of SOD from the principal species of fish examined and their pI values are shown in Figure 6 and Table 2, respectively. No SOD polymorphism was observed within any of the different species examined. For example, the electrofocusing pattern obtained from sea trout caught in North Wales was identical to that obtained from fish caught in Cornwall. Similarly, brown trout caught in Cumbria had an identical pattern to brown trout held at any of the fish farms covered in this work.

Although no common polymorphic forms were observed within a species, it was frequently very difficult to differentiate certain species because the SOD isoenzymes had very similar pI values. Obvious examples are the roach (5.08), chub (5.06), pike (5.04), dace (5.05), and brook trout (5.08). Although grayling and crucian carp could not be separated immunologically they

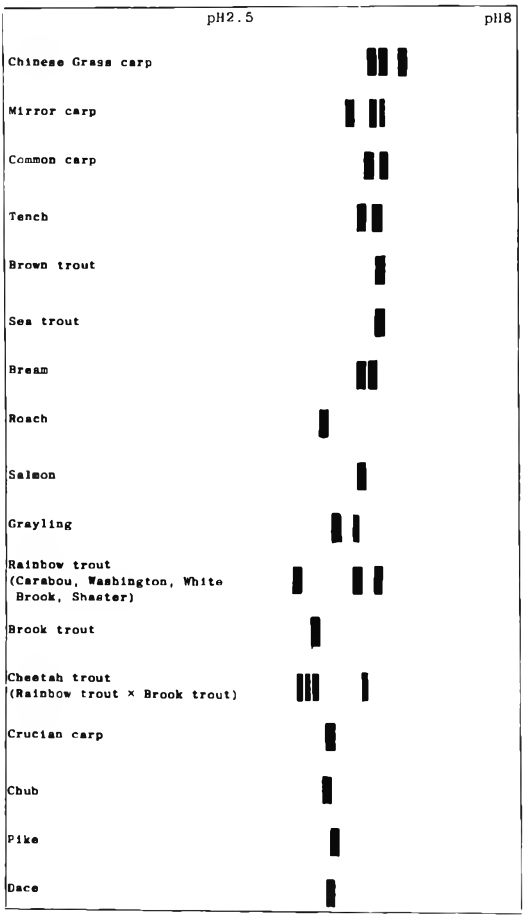

Figure 6. Isoelectric focusing patterns of SOD isoenzyme of various fish species on pH 2.5-8 gradient.

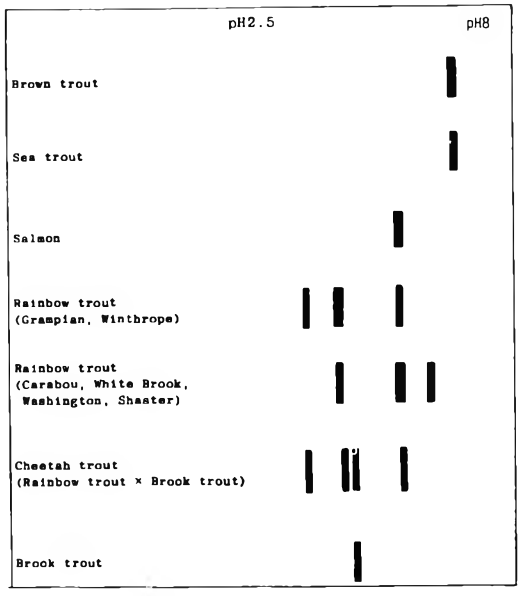

Figure 7. Isoelectric focusing patterns of SOD isoenzymes from the family Salmonidae on pH 2.5-8 gradient.

could be differentiated from members of the Salmonidae by using the electrofocusing techniques (Figure 6).

Figure 7 shows the electrofocusing patterns of the members of the Salmonidae family that were examined. We found that differentiation was possible with the exception of the sea trout and brown trout (pI 4.76), which are considered by many authorities to be of common ancestry (24).

The rainbow trout could be classified into 2 distinct polymorphic forms depending on the pI values of their isoenzymes. The Carabou, Washington, Shaster, and White Brook strains all showed 3 isoenzymes whose pI values were 4.80, 4.90, and 5.07, respectively. Although the intensity of the isoenzymes from the Carabou, Washington, and Shaster strains appeared identical, the 2 most anodic isoenzymes from the White Brook strains were of a much higher intensity.

The remaining strains, that is, the Winthrope and Grampian, also possessed 3 isoenzymes, 2 of which had identical pI values to the most cathodic isoenzymes observed in the Carabou, Shaster, Washington, or White Brook strains. The third isoenzyme in this complex has a pI value of 5.28 which was the most cathodic isoenzyme recorded from any member of the Salmonidae family studied. The majority of the rainbow trout examined were of an unknown strain and came from various fish farms who sold fish directly to the public for consumption. In each case the patterns obtained were identical to the Winthrope or Grampian strains.

The cheetah trout was the only hybrid species examined. It was produced from a cross between rainbow trout and the American brook trout (char) and is used primarily as a game fish. The electrofocusing patterns that we obtained appeared to be a mixture composed of the triple banded pattern associated with a Grampian or Winthrope strain and the single component found in the brook trout. This pattern was the most complex observed in any member of the Salmonidae family examined.

Tissue Studies

The electrofocusing patterns obtained from the

Table 2. Mean pI values for SOD isoenzymes found in red cells of Salmonidae and members of 2 coarse fish families [a]

Species	Mean pI values for each SOD isoenzyme	Std dev.	No. of detns
Chinese grass carp	4.52 4.66 4.75	0.05 0.10 0.12	5
Mirror carp	4.72 4.79 4.88	0.04 0.02 0.17	5
Common carp	4.72 4.79	0.04 0.01	5
Crucian carp	5.00		5
Tench	4.79 4.86	0.6 0.05	5
Roach	5.08	0.1	5
Bream	4.84	0.14	17
Dace	5.05	0.10	9
Chub	5.06		3
Pike	5.04	0.05	3
Grayling	4.87 4.94	0.02 0.05	10
Salmon	4.89	0.05	26
Sea trout	4.78	0.10	26
Brown trout	4.76	0.09	140
Brook trout	5.08	0.06	10
Rainbow trout strain:			
Carabou	4.80 4.90 5.07	0.02 0.03 0.04	4
Washington			3
White brook			4
Shaster			4
Grampian	4.89 5.09 5.28	0.12 0.08 0.10	4
Winthrope			17
Unknown strains			36

[a] The electrofocusing gradient was linear from pH 4.25 to 5.25 (approximately).

samples of rainbow trout liver showed that they were identical to those from the red cell except that an additional, but fainter, band was observed focusing in a more cathodic position to each of the main isoenzymes. The electrofocusing patterns that we obtained from roe, although identical to those in blood, were a great deal fainter and did not appear to be accompanied by additional satellite isoenzymes. Finally, no SOD activity was observed in the tissue associated with the few grayling scales examined.

Conclusion

The results from this study indicate that it is possible to differentiate various members of the Salmonidae family on the basis of the isoenzymes of SOD from the red cell. In addition, we obtained some evidence which indicated that an identical polymorphism could be found in various tissues of the rainbow trout, although the appearance of additional but fainter bands of activity might be regarded as further evidence for an additional locus as suggested by Taggart et al. (11).

Although some 28 species of fish (excluding rainbow trout variants) from 8 different orders have been examined, they have been few in number with the possible exception of the brown and sea trout. In forensic work, it would therefore be advisable to use control samples from the crime area whenever possible in case rare variants, hitherto unreported, are discovered.

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MICROBIOLOGICAL METHODS

Comparison of Iron Milk and Official AOAC Methods for Enumeration of *Clostridium perfringens* from Fresh Seafoods

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Fresh seafoods collected from Seattle area markets were evaluated for the presence and numbers of *Clostridium perfringens*. In a comparison of the official AOAC Shahidi-Ferguson perfringens and the simple iron milk methods, 29 of 287 samples of fresh fish and shellfish were positive for *C. perfringens*. All 29 positive samples were detected by the iron milk medium; however, only 7 of the 29 were detected by the AOAC methods. A statistical evaluation of the data showed a significant difference between the 2 methods.

Of several selective differential media devised for the enumeration and isolation of *Clostridium perfringens*, some of those commonly used are Shahidi-Ferguson perfringens (SFP) (1), sulfite-polymyxin-sulfadiazine (SPS) (2), tryptose-sulfite-neomyxin (TSN) (3), and tryptose-sulfite-cycloserine (TSC) (4). These media contain sulfite and at least one antibiotic. The selectivity and ease of detection are based on the antibiotic's inhibition of competing facultative organisms and the reduction of sulfite to sulfide which combines with iron, producing black colonies. However, because the procedures for preparing the selective media are costly, time-consuming, and tedious, a simple and rapid method for the isolation and enumeration of *C. perfringens* in foods is needed.

Rapid screening procedures (5, 6) have been developed for the enumeration of *C. perfringens* in foods. The rapid perfringens medium (RPM), developed by Erickson and Diebel (5), consists of fortified litmus milk broth with inhibitory agents, polymyxin B sulfate, and neomycin sulfate. In a comparison with SPS and TSC plate media, the RPM was superior in selectivity and sensitivity, detecting low to moderate numbers of *C. perfringens*. Smith (7) demonstrated that inhibitors slowed the growth of certain strains of *C. perfringens* as well as competing facultative organisms.

The method developed by St. John et al. (6) used simple iron milk medium. The selectivity of this medium relied solely on the rapid growth of *C. perfringens* at 45°C and the typical stormy fermentation reaction within 18 h. The present study compared the simple iron milk most probable number (MPN) procedure (6) with the official AOAC method (8) for the isolation and enumeration of *C. perfringens* from fresh seafoods.

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Experimental

Samples.—A total of 287 fresh seafood samples were collected from Seattle retail markets.

Media.—The iron milk medium (6) was prepared by placing 10 mL homogenized pasteurized milk and 0.2 g iron powder in 16 × 150 mm screw-cap tubes. The medium was sterilized at 10 lb pressure, 116°C, for 10 min and used on the day of preparation. The MPN procedure was used as outlined by St. John et al. (6). The official AOAC method (8), using the Shahidi-Ferguson perfringens direct spread plate (1), was followed. The medium was prepared according to label instructions (Difco) and used only on the day of preparation. Both media were tested for growth by inoculation with *C. perfringens* (ATCC 13124).

Biochemical tests.—Samples exhibiting stormy fermentation in iron milk after 16–18 h at 45°C were streaked on SFP plus egg yolk without antibiotics and incubated anaerobically at 37°C for 24–28 h. Black colonies displaying lecithinase were picked to freshly steamed tubes of cooked meat medium for holding. Isolates were tested for motility, nitrate reduction, gelatin liquefaction, and lactose fermentation, as outlined in *Official Methods of Analysis* (8).

Results

A breakdown of the total numbers and types of samples tested is given in Table 1. Each of the 287 samples was analyzed for the presence of *C. perfringens* by the 2 test methods (Table 2). The distribution ranges and percentages of positive samples for *C. perfringens* are given for the iron milk and the SFP media. Of 287 samples ana-

Table 1. Common and scientific names of seafood species analyzed

Common name	Scientific name	No. of units tested
Roundfish		
True cod	*Gadus macrocephalus*	11
Ling cod	*Ophiodon elongatus*	16
Black cod	*Anoplopoma fimbria*	24
Rockfish (red snapper)	*Sebastes* species	49
Flatfish		
Dover sole	*Microstomus pacificus*	21
Petrale sole	*Eopsetta jordani*	7
English sole	*Parophrys vetulus*	3
Rex sole	Not identified	7
Halibut	*Hippoglossus stendepis*	14
Starry flounder	*Platichthys stellatus*	3
Crustacean		
Oysters	*Crassostrea gigas*	18
Clams	*Saxidomus giganteus*	12
Mussels	*Mytilus edulis*	15
Scallops	*Patinopecten caurinus*	14
Squid	*Loligo opalescen*	17
Molluscan		
Dungeness crab	*Cancer magister*	21
Shrimp	*Pandalus jordani*	35

lyzed, 29 (10.1%) were positive for *C. perfringens* in the iron milk method and 7 (2.4%) were positive in SFP, giving a percent-positive ratio of 4.2 to 1.0. The 7 samples positive by the SFP method were also positive by the iron milk MPN method. In shellfish and finfish the percent positive was 18.2 and 3.2, respectively, in the iron milk method and 4.6 and 0.6, respectively, in the SFP method. These values show better recovery of *C. perfringens* in the iron milk method than in the SFP plating medium.

Of the seafoods examined, the molluscan shellfish were contaminated at a higher rate than the crustaceans. With the iron milk method, *C. perfringens* was isolated from 23 of 76 (30.3%) of the molluscs tested, whereas only 5 of 76 (6.6%) were positive with the SFP method. In each of the seafoods examined, the percent positive in the iron milk method was equal to or greater than that in the SFP method.

The average geometric means of 287 samples in the iron milk medium were 1.4/g and 1.1/g in the iron milk and SFP methods, respectively. The microbial count range was similar in both media with <3–43/g obtained in iron milk and <10–60/g in SFP. Fish containing *C. perfringens* and the percentage of positive samples for the iron milk method were: roundfish (cod, etc.) and spiny rockfish, 2.0%; flatfish (sole, flounder, and halibut), 5.5%; shrimp, 5.6%; oysters, 38.9%; mussels, 53.3%; and clams, 66.6%. The percentage of positive samples by the SFP method were flatfish, 1.8%; shrimp, 5.6%; clams, 8.3%; oysters, 11.1%; and mussels, 13.3%. These data clearly indicate that use of the iron milk MPN procedure increases the number of *C. perfringens* recovered from seafood. These data also show that the filter-feeding shellfish (clams, oysters, and mussels) contain more *C. perfringens* than do roundfish and flatfish. The flora in the filter feeders is indicative of water conditions in the growing area. With other fish, the flora is indicative of handling and processing conditions.

In this study, as in the study by St. John et al. (6), all tubes of iron milk medium showing a positive reaction were subjected to further testing for the presence of *C. perfringens*. In 63 positive tubes showing typical stormy fermentation at 45°C within 18 h, *C. perfringens* was confirmed in 57 tubes (90%). Tubes that were negative for *C. perfringens* and read as false positive had all been

Table 2. Comparison of iron milk and Shahidi-Ferguson perfringens (SFP) methods for enumeration of *C. perfringens* in fresh seafoods[a]

Seafood	No. of samples	SFP		Iron milk	
		No. of positive samples (%)	Count range/g	No. of positive samples (%)	Count range/g
Roundfish	51	0		1 (2.0)	3.6
Rockfish	49	0		1 (2.0)	3.6
Flatfish	55	1 (1.8)	10	3 (5.5)	3.6–23
Oysters	18	2 (11.1)	40–60	7 (38.9)	3.6–43
Clams	12	1 (8.3)	10	8 (66.6)	3.6–23
Mussels	15	2 (13.3)	10–50	8 (53.3)	3.6–43
Shrimp (unpeeled)	18	1 (5.6)	20	1 (5.6)	15
Total shellfish	132	6 (4.6)	10–60	24 (18.2)	3.6–43
Total finfish	155	1 (0.6)	10	5 (3.2)	3.6–23
Total seafoods	287	7 (2.4)	10–60	29 (10.1)	3.6–43

[a] Dungeness crab, shrimp (peeled), scallops, and squid.

incubated well beyond the 16–18 h prescribed by St. John et al. (6) and thus further reinforce their caution against extended incubation. Isolates selected from the SFP medium were confirmed in only 7 to 10 samples (70%) showing typical reactions. All samples that were positive in SFP medium were positive in the iron milk medium.

The count data obtained from 287 samples by the 2 test media were subjected to the standard Z-test (difference between 2 proportions) (9). A Z-value of 3.51 was calculated, indicating a highly significant difference between the counts.

Discussion

To the regulatory and quality control microbiologist, a rapid, simple, and accurate method for the enumeration and isolation of *C. perfringens* is necessary. Several investigators (5, 6, 10) have developed methods useful to field microbiologists. To circumvent the problem of using a plate medium with an inhibitory agent and avoid laborious, costly, and time-consuming preparation of plates, St. John et al. (6) used pasteurized whole milk supplemented with 0.2% iron powder in the direct enumeration of *C. perfringens*. They concluded that compared with 3 plating media, the milk MPN method gave the highest confirmation for all isolates tested. Erickson and Diebel (5) showed the RPM to be superior to SPS in detecting *C. perfringens* from naturally contaminated food products. Of 774 samples tested, they found 71% positive in RPM and 22% positive in SPS. These data clearly indicated the effectiveness of the milk containing RPM; however, 11 ingredients are required in the initial preparation of the RPM medium, making it complex and time-consuming.

Stormy fermentation in iron milk and RPM is highly indicative of the presence of *C. perfringens* (5, 6). The stormy fermentation, i.e., the production of an acid curd with subsequent disruption of the curd with large volumes of gas, is produced only by *C. perfringens* at 45°C within 18 h. St. John et al. (6) reported that organisms such as *Escherichia coli* and *Bacillus* species can produce reactions similar to stormy fermentation but at a lower rate than *C. perfringens*. The acid clot is produced by the acidification of milk (lactic acid fermentation) (11) to a pH of 4.5. The lactic acid denatures the casein to yield a precipitate. At pH 4.5, the numbers of *C. perfringens* are reduced upon standing; therefore, to obtain viable *C. perfringens* and to prevent false positives, it is recommended that results be read and cultures transferred after 16–18 h of incubation.

In conclusion, the iron milk MPN method was superior to the official AOAC direct plate method in sensitivity and selectivity for the isolation and enumeration of *C. perfringens* from naturally contaminated seafoods. In addition, the simple preparation of the medium and evaluation of the reactions make the iron milk method very useful for rapid evaluation of fresh seafood samples in the field laboratories.

Acknowledgment

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DRUGS

Ion-Pair Partition Chromatography of Mefenamic Acid with Tetraalkylammonium Cations: Development of Analytical Method from Extraction Data

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The ion-pair partition properties of mefenamic acid with the methyl, ethyl, *n*-propyl, and *n*-butyl homologs of tetraalkylammonium cation were studied in relation to a model of an assay procedure for acidic drugs. Variables studied included identity and concentration of pairing ion and composition of extracting solvents. Resulting data were used to develop a partition chromatographic assay procedure. Standard recoveries averaged 99.01 ± 0.82%. Assays of commercial capsules were reproducible.

Mefenamic acid, an analgesic for mild to moderate pain, including primary dysmenorrhea, is thought to act by stopping the pharmacological actions and biosynthesis of prostaglandins.

The literature cites many examples of clinical assay and toxicological screening procedures for mefenamic acid, but few are applied to pharmaceutical formulations. Devaux et al. (1) reported derivatization reactions with visible or fluorescence spectroscopic detection. Iwai et al. (2) and Agrawal (3) reported potentiometric titration procedures. Vdoviko et al. (4) described a polarographic method. The *British Pharmacopoeia* prescribes a titration for capsules and bulk material (5). All of these procedures are either nonspecific or require extensive sample pretreatment; none was judged suitable for analysis of pharmaceutical dosage forms.

Ion-pairing column partition chromatography (IP-CPC) has been widely used for the determination of both acidic and basic drugs in pharmaceutical formulations (6). The technique is well defined and often produces a method which is both rugged and highly selective. The partition can be precisely studied through simple distribution experiments.

We report a detailed study of the ion-pair partition of mefenamic acid with the methyl, ethyl, *n*-propyl, and *n*-butyl homologs of tetraalkylammonium cation, R_4N^+, using mixtures of chloroform and isooctane as the extracting or eluting solvent. Data derived from this study were used to develop an IP-CPC assay procedure, which was applied successfully to standard solutions and to commercial dosage forms.

Experimental

Apparatus and Reagents

(a) *Chromatographic tube and tamping rod.*—See 37.002(a) and (b) (7).

(b) *UV spectrophotometer.*—Cary 118 scanning UV/VIS spectrophotometer, or equivalent.

(c) *Solvents.*—Reagent grade chloroform (water-saturated) and isooctane.

(d) *Pairing ions.*—25% aqueous solution of *n*-Pr_4NOH (Eastman Kodak Co., Rochester, NY 14650), or equivalent. *0.001M solution:* Dilute 1 mL 25% solution to 100 mL with 0.1N NaOH; then dilute 10 mL of this solution to 100 mL with 0.1N NaOH. *10% solution:* Dilute 10 mL 25% solution to 25 mL with 0.1N NaOH.

(e) *Mefenamic acid.*—Pharmaceutical reference material used without purification (Parke, Davis & Co., Detroit, MI 48232).

(f) *Acidic MeOH (1%).*—1 mL concentrated HCl/100 mL MeOH.

(g) *Acidic chloroform.*—5 mL 1% acidic MeOH/100 mL chloroform.

(h) *Prewash.*—Chloroform–isooctane (1 + 2).

(i) *Celite 545.*—Acid-washed (Johns-Manville Corp., Denver, CO 80217).

Sample Preparation

Dissolve a sufficient number of capsules in 0.1N NaOH to produce a 5 mg/mL solution of mefenamic acid. Dissolve by mechanical shaking for 2 h or by using an ultrasonic bath for 30 min. Before sampling, let particulates settle, or filter through 0.6 μm filter.

Standard Solution

Dissolve an accurately weighed sample of mefenamic acid in sufficient acidic chloroform to obtain a concentration of ca 0.025 mg/mL.

Column Preparation

In small beaker, mix 3 mL 0.001M *n*-Pr_4NOH with 4 g Celite 545. Transfer to chromatographic tube containing glass wool pledget in base, and

tamp to uniform mass. Similarly, combine 0.1 mL 10% n-Pr_4NOH and 1.0 mL sample preparation. Mix completely by gently swirling. Add 2 g Celite, mix, and transfer to tube. Scrub beaker with 1 g Celite containing 3 drops 0.1N NaOH and transfer Celite to tube. Wipe utensils with glass wool pledget. Place pledget atop column and tamp column to uniform mass.

Determination

Rinse beaker and utensils with 75 mL prewash. Pass prewash through column and discard eluate. Elute column with approximately 175 mL chloroform. Collect eluate in 200 mL volumetric flask containing 10 mL acidic MeOH. Rinse tip of column with chloroform into flask. Dilute eluate to volume with chloroform. Concomitantly determine absorbances of both sample and standard solutions in 1 cm quartz cuvets at wavelength of maximum absorbance (ca 353 nm), using acidic chloroform as blank.

Calculate quantity (mg) of mefenamic acid in the 1.0 mL portion of sample preparation taken by the formula

$$\text{Mefenamic acid (mg)} = (A_u/A_s) \times 200C_s$$

where A_u and A_s = absorbances of sample and standard solutions, respectively; and C_s = concentration of standard solution (mg/mL).

Extraction Study

Tetraalkylammonium hydroxide (R_4NOH) reagents were obtained as 10 or 25% aqueous solutions and standardized when necessary by titrating with aqueous HCl.

Extent of extraction, $\%_o$, was calculated by comparison of the aqueous phase before extraction with either the aqueous phase after extraction or the aqueous portion of a 0.1N NaOH back extraction of the organic phase. The distribution ratio D was then calculated from

$$D = (\%_o/\%_w) \times (V_w/V_o) \qquad (1)$$

where $\%_o$ and $\%_w$ = percentages of the drug in the organic and aqueous phases, respectively; and V_w and V_o = respective volumes of the aqueous and organic phases. Volume ratio, V_w/V_o, was adjusted to obtain a $\%_o$ value of ca 50 for the ion-paired drug because this value produces D values of optimum accuracy and precision. Absorbances used in calculations were differences between an absorbance maximum (ca 285 nm) and a minimum (ca 317 nm).

The ion-pair extraction constant K_{QA} and solvation number N were calculated from

Table 1. Extraction constants [a] for chloroform extractions of mefenamic acid as ion-pairs with tetraalkylammonium cations (R_4N^+) in 0.1N NaOH

R	K_{QA}	log K_{QA}	Δlog K_{QA}
n-Butyl	7.2×10^4	4.9	–
n-Propyl	4.0×10^2	2.6	2.3
Ethyl	4.7	0.7	1.9
Methyl	1.4×10^{-1}	−0.9	1.6

[a] Average of multiple experiments: 4 for n-butyl, 3 for n-propyl, 13 for ethyl, and 2 for methyl.

$$D = K_{QA}(Q^+)_w Y^N \qquad (2)$$

where $(Q^+)_w$ = molar concentration of the pairing ion in the aqueous phase after extraction; and Y = volume fraction of chloroform in the organic phase.

Results and Discussion

Mefenamic acid is practically insoluble in water, sparingly soluble in ether and chloroform, and freely soluble in aqueous base (pKa = 4.2) (8). By choosing a solution with a pH much greater than the pKa value, complete ionization is assured and free acid extraction is insignificant. Under these conditions, the distribution D is defined in accordance with principles previously developed (9–11) by the equation

$$D = (QA)_o/(A^-)_w = K_{QA}(Q^+)_w Y^N \qquad (3)$$

where (QA) = concentration of solvated ion-pair complex; (A^-) = concentration of ionized drug; and subscripts w and o refer to the aqueous and organic phases, respectively. Note that D is independent of drug concentration. The validity of this equation in the present instance was verified in detail experimentally.

Pairing Ions

Table 1 records the observed extraction constants for chloroform extractions of mefenamic acid paired with 4 homologs of R_4N^+. Each value represents an average of multiple experiments: 2 for methyl, 13 for ethyl, 3 for n-propyl, and 4 for n-butyl.

It was observed that K_{QA} increases regularly with the length of the carbon chain in R_4N^+ and shows a very large change—a factor of approximately 500 000—between the methyl and n-butyl homologs. A plot of log K_{QA} vs carbon number is linear (slope = 1.91; corr. coeff. = 0.996). Doyle and Barkan (12) made similar observations for an acid extracted with the same homologs of R_4N^+ as did Gustavii (13), who ex-

Table 2. Linear dependence of distribution on pairing ion concentration at constant mefenamic acid concentration for chloroform extractions with tetrapropylammonium cation [a]

$(Q/A)^b$	$(Q^+)^c$	D	K_{QA}
75.8	12.10	4.76	393
51.6	8.42	3.42	406
22.8	3.49	1.61	461
6.3	1.05	0.43	410
3.9	0.69	0.32	464
1.1	0.21	0.10	476
0.13	0.035	0.017	486

[a] Plot of D vs (Q^+) gave slope K_{QA} = 395, corr. coeff. = 0.999.

[b] Molar ratio of pairing ion to drug in aqueous phase after extraction.

[c] Final pairing ion concentration × 10^{-3}M.

Table 3. Solvent effects in extractions of mefenamic acid as ion-pairs with tetrapropylammonium cation [a]

log D	Y^b	log Y
1.12	1.00	0.000
1.03	0.90	−0.046
0.52	0.80	−0.097
0.18	0.70	−0.155
−0.19	0.60	−0.222
−0.89	0.50	−0.301
−1.16	0.40	−0.398

[a] Plot of log D vs log Y gave slope N = 6.19, corr. coeff. = 0.991.

[b] Y = volume fraction of chloroform in isooctane.

tracted amines with quaternary ammonium picrates.

Doyle and Proctor (11) found that polar groups oriented *ortho* to the ion-pair site of phenols (—OH) had only a slight effect on the solvation number N, but drastically affected K_{QA}. However, in the present study, the size and correlation of K_{QA} values for R_4N^+ with mefenamic acid show little or no effect from the presence of a polar —NHAr group oriented *ortho* to the carboxylic acid ion-pairing site.

Table 2 records observed distribution values for chloroform extractions of mefenamic acid as ion-pairs with *n*-Pr_4N^+. Duplicate 0.1N NaOH solutions with constant drug concentration (3.5 × 10^{-4}M) and varying pairing ion concentration (initially 0.01 to 0.0001M) were extracted with chloroform. Values for V_w/V_o suitable for the determination were selected by estimation from Equation 3 (Y = 1) and from Table 1. Observed D values correlated directly to the pairing ion concentration (Equation 3, corr. coeff. = 0.999) with a slope (K_{QA} = 395) comparable to the value obtained independently in Table 1 (K_{QA} = 404).

Solvent Effects

It has been shown that diluting chloroform with a nonpolar solvent (in this case isooctane) produces a large and predictable effect on the ion-pair partition of acids (12, 14) and amines (15, 16). Doyle and Proctor (11) showed that this "solvent effect" made possible highly selective chromatographic partitions.

Table 3 records values of D for extractions of mefenamic acid as ion-pairs with *n*-Pr_4N^+. Aliquots of a 0.1N NaOH solution containing a constant ratio of drug to pairing ion were extracted with an organic solvent containing from 100% (Y = 1.00) to 40% (Y = 0.40) chloroform in isooctane. By plotting log values of these data, a value for exponent N in Equation 3 is obtained. This value, termed the solvation number by Higuchi (16), is thought to represent the number of polar solvent molecules associated at the anion charge site of the ion-pair. Although this explanation is disputed by Frieser (17), experience has proven its general empirical applicability to ion-pair systems. Higuchi et al. (16) reported integral values of 5 for N, whereas this laboratory has observed values ranging from 4.5 to 6.5. The observed value (N = 6.2) is large, but well within the expected range. This large solvation number, which indicates a pronounced sensitivity of D to solvent polarity, contributes to the separation specificity of IP-CPC.

Selection of Chromatographic Conditions

Considering the above discussion, it is apparent that the partition of mefenamic acid is precisely described by the distribution as defined in Equation 3. It is also apparent that numerous combinations of $(Q^+)_w$ and Y which produce acceptable partitions are possible. Partition chromatographic systems commonly used in this laboratory require D to be greater than 0.06 and less than 0.01 for complete elution and retention, respectively. Calculations employing data from Tables 1 and 3 in Equation 3 indicate that *n*-Pr_4N^+ is the optimum reagent for chromatographic isolation of mefenamic acid.

Method

Separation is obtained by controlling the organic solubility of the ion-paired drug. An alkaline solution of the drug is ion-paired by adding excess *n*-Pr_4NOH, which is mixed with a solid adsorbent and placed atop another ad-

Table 4. Standard recoveries of mefenamic acid solutions

Sample, mg[a]	Rec., mg	Rec., %
6.418	6.363	99.14
	6.383	99.45
	6.315	98.40
	6.390	99.56
	6.390	99.56
5.028	4.998	99.40
	4.985	99.14
4.990	4.843	97.05
	4.958	99.36
Av. ± SD		99.01 ± 0.82

[a] Amount (mg) mefenamic acid (actual) placed on column.

Table 5. Duplicate assays of 4 separate mefenamic acid capsule [a] solutions

Sample, 5.00 mg[b]	
Found, mg	Percent of label claim
4.67	93.4
4.75	95.0
4.95	99.0
4.99	99.8
4.90	98.0
4.90	98.0
4.66	93.2
4.66	93.2

[a] 250 mg mefenamic acid/capsule.
[b] Amount (mg) mefenamic acid (per label claim) placed on column.

sorbent layer containing a precisely known quantity of pairing ion. The column is then prewashed with chloroform diluted with sufficient isooctane to reduce D to less than 0.01, a value which in this case corresponds to 33% chloroform in the prewash. Excipients which could produce spectral interferences are thus removed. The drug is retained in the bottom (trap) layer. Finally, a more polar solvent, pure chloroform, increases D sufficiently to permit elution of the ion-pair from the column into a volumetric collector containing a quantity of strong acid. HCl, dissolved in methanol to give it miscibility with chloroform, breaks up the ion-pair whose spectrophotometric properties differ from the free acid. Mefenamic acid is then determined spectrally in chloroform solution without spectral interference.

Minimum effort in sample preparation is recommended. Capsule contents rapidly dissolve once the gelatin capsule has disintegrated. Two hours or less of mechanical shaking or 30 min in an ultrasonic bath is sufficient. After several hours, solutions turn from yellow to bright orange. Excessive mechanical shaking or ultrasonification results in an acceleration of the color change. Some samples gave low yields after this transformation. A white precipitate, thought to be TiO_2, may be removed with a 6 μm filter or allowed to settle out before sampling. Presence or absence of the precipitate had no effect on yields.

Tables 4 and 5 record the results of recoveries of the standard and assays of commercial capsules, respectively. Recoveries of the standard averaged 99.01 ± 0.82% for 9 determinations taken from 3 different solutions prepared at different times over a period of several weeks. Duplicate assays of 4 separate capsule solutions were made. Although results varied between solutions, the variation within the duplicates was small to nil.

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High Pressure Liquid Chromatographic Determination of Sulfisoxazole in Dosage Forms: Collaborative Study

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A reverse phase high pressure liquid chromatographic method for the determination of sulfisoxazole in tablets, solution, and ointment dosage forms was collaboratively studied by 7 laboratories. The method uses a C_{18} column, an acetonitrile-acetic acid-water mobile phase, sulfadimethoxime as the internal standard, and photometric detection at 254 nm. Collaborators were supplied samples of 2 commercial tablets, 1 synthetic tablet powder, 1 commercial and 1 synthetic ophthalmic solution, and 1 commercial ophthalmic ointment. Mean recoveries of sulfisoxazole from the synthetic tablet powder and ophthalmic solution were 99.9 and 100.0%, respectively. Mean coefficients of variation for all samples analyzed were less than 2%. The method was adopted interim official first action.

Prior work from this laboratory resulted in a reverse phase high pressure liquid chromatographic (HPLC) method for the assay of sulfisoxazole in dosage forms (1). Results obtained with this HPLC method showed good agreement with those by the titrimetric and colorimetric methods of *U.S. Pharmacopeia XX* (2). Because the HPLC method is stability-indicating and offers a wider applicability and greater convenience in the analysis of sulfisoxazole in dosage forms, a collaborative study was undertaken. Some minor modifications were introduced into the original method, however, i.e., the internal standard was changed from sulfabenzamide to sulfadimethoxine to improve its resolution from the analyte, and the final dilution of the sample was done with mobile phase rather than with methanol to preclude sample solvent-mobile phase overload during the development.

Collaborative Study

Collaborators from 7 laboratories received 6 samples consisting of 4 commercial and 2 synthetic formulations, and were requested to assay each of them in duplicate. Each collaborator was supplied the theoretical average weight of the synthetic tablet powder, so that values could be reported as mg/tablet. The collaborators were also furnished an instruction sheet, a copy of the method, and reporting forms. No restriction was placed on the source of the chromatographic column needed in the study.

Sulfisoxazole in Tablets, Solutions, and Ointments

Liquid Chromatographic Method

Interim Official First Action

37.D06 ***Principle***

Sulfisoxazole content of tablets, solns, and ointments is detd by reverse phase HPLC using ternary aq. mobile phase, UV detection at 254 nm, and sulfadimethoxine as internal std.

37.D07 ***Apparatus***

(a) *Liquid chromatograph.*—DuPont Model 841 solv. pump equipped with 254 nm detector (E. I. duPont de Nemours and Co.), 10 μL injection valve (Valco Instruments Inc., PO Box 55603, Houston, TX 77055), and Model 3380A integrator (Hewlett-Packard). Equiv. HPLC system and strip chart recorder may be used.

(b) *Chromatographic column.*—Stainless steel, 30 cm × 3.9 mm id, packed with 10 μm μBondapak C_{18} (Waters Associates, Inc.) or equiv. meeting appropriate HPLC system suitability requirements.

37.D08 ***Reagents***

(a) *Solvents.*—UV grade MeOH and *n*-heptane (Fisher Scientific Co., or equiv.), and acetonitrile (Burdick & Jackson Laboratories, Inc., or equiv.).

(b) *Mobile phase.*—Acetonitrile-acetic acid-H_2O (30 + 1 + 69); flow rate 2.0 mL/min. Re-

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tention times: sulfisoxazole, ca 4 min; internal std, ca 5.5 min. Vary ratio of acetonitrile to H_2O to meet HPLC system suitability requirements. Increased acetonitrile decreases retention time.

(c) *Internal std soln.*—Dissolve 80 mg USP Ref. Std Sulfadimethoxine in MeOH and dil. to 100 mL with MeOH.

(d) *Sulfisoxazole std soln (5.0 mg/100 mL).*—Transfer 100 mg accurately weighed USP Ref. Std Sulfisoxazole (previously dried 2 h at 105°) to 100 mL vol. flask and dil. to vol. with MeOH. Transfer 5.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix.

37.D09 *Sample Preparation*

(a) *Tablets.*—Det. av. wt and grind to pass No. 60 sieve. Transfer accurately weighed portion of powd. equiv. to 500 mg sulfisoxazole to 100 mL vol. flask, add 25 mL MeOH, stopper, mix on mech. shaker 30 min, dil. to vol. with MeOH, mix, and filter. Transfer 20.0 mL aliquot of filtrate to 100 mL vol. flask and dil. to vol. with MeOH. Transfer 5.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix.

(b) *Liquids (injections and ophthalmic solns).*—Accurately transfer vol. of dosage form contg ca 200 mg sulfisoxazole to 200 mL vol. flask and dil. to vol. with MeOH. Transfer 5.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix.

(c) *Ointments.*—Transfer accurately weighed amt of sample (S) contg ca 50 mg sulfisoxazole to 125 mL separator contg 50 mL *n*-heptane, shake to disperse ointment, and ext with three 25 mL portions of MeOH–H_2O (2 + 1), passing each ext consecutively thru second 125 mL separator contg 50 mL *n*-heptane. Collect exts in 100 mL vol. flask and dil. to vol. with MeOH. Transfer 10.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix.

37.D10 *Determination*

Equilibrate column with mobile phase at flow rate of 2 mL/min. Make 3 replicate injections of sulfisoxazole std soln. Using either peak area or peak ht measurements, for each injection det. response ratio of sulfisoxazole to internal std. In suitable system, capacity factor, k', for sulfisoxazole should be 1.0–3.0; resolution factor, R, for sulfisoxazole peak and internal std peak should be ≥ 1.5; and coefficient of variation of response ratio for 3 replicate injections each of sample and sulfisoxazole std working solns should be $\leq 2.0\%$. When chromatgc requirements are met, make alternate injections of std and sample solns, and det. response ratio for each. Retention times for sulfisoxazole and internal std must be same for sample and std soln injections.

37.D11 *Calculations*

Calc. content of sulfisoxazole in dosage form as follows:

Tablets: $\text{mg/tablet} = (RR/RR') \times C \times (T/S) \times 100$

Solns: $\text{mg/mL} = (RR/RR') \times (C/V) \times 40$

Ointments: $\text{mg/g} = (RR/RR') \times (C/S) \times 10$

where RR and RR' = response ratio of sample and std solns, resp.; C = amt sulfisoxazole in 100 mL final std soln, mg; T = av. tablet wt, g; S = sample wt, g; and V = vol. soln taken, mL.

Results and Discussion

The collaborators were directed to use a C_{18} column measuring 25–30 cm long × 4.0–4.6 mm id, and were permitted to vary the proportion of acetonitrile to allow capacity and resolution system suitability requirements to be met. The collaborators were requested to use peak height in the calculations. However, 4 of the 7 collaborators chose to measure peak areas, using an electronic integrator.

Table 1 summarizes the HPLC conditions used by the 7 collaborators. None of the collaborators reported any problems with the proposed HPLC method and none offered suggestions for procedural changes. A chromatogram of a standard mixture of sulfisoxazole and sulfadimethoxine, the internal standard, obtained during the collaborative study is shown in Figure 1. The small peak retained at about 1.5 min was due to the presence of methanol in the sample solution but not in the mobile phase.

The quantitative data submitted by the 7 collaborating laboratories along with the average concentration, average percentage of declared, standard deviation, and coefficient of variation for each of the samples are shown in Table 2. All assay results were obtained in duplicate except those of collaborator F, who submitted a single result for the synthetic tablet powder. Therefore, the statistical data for this sample are based on 13 measurements, compared with 14 for the

Table 1. HPLC conditions of collaborative study for sulfisoxazole

Coll.	Column	Mobile phase (acetonitrile–acetic acid–water)	k'[a]	R[b]	Calcn mode
A	μBondapak C_{18}	25 + 1 + 74	1.3	2.7	peak area
B	Ultrasphere ODS	30 + 1 + 69	2.1	3.0	peak ht
C	Whatman ODS-3	36 + 1 + 63	2.4	3.2	peak area
D	μBondapak C_{18}	25 + 1 + 74	2.2	2.6	peak area
E	μBondapak C_{18}	28 + 1 + 71	2.0	2.5	peak ht
F	μBondapak C_{18}	30 + 1 + 69	3.9	2.8	peak area
G	μBondapak C_{18}	30 + 1 + 69	2.0	3.2	peak ht

[a] Capacity factor for sulfisoxazole.
[b] Resolution factor of sulfisoxazole and sulfadimethoxine internal standard.

other 5 formulations. Recoveries of sulfisoxazole from the synthetic tablet powder (99.9%) and from the synthetic solution (100.0%) were excellent. The corresponding coefficient of variation values for these 2 samples were 1.34 and 1.98%, respectively. Although no synthetic ointment sample was submitted to collaborative study, an earlier intralaboratory evaluation of this formulation (1), using the original HPLC method, yielded results which were within 1% of those obtained by the colorimetric method of *U.S. Pharmacopeia XX* (2). The two 500 mg commercial tablet samples, the 40 mg/mL commercial ophthalmic solution, and the 40 mg/mL

Table 2. Collaborative results of the HPLC determination of sulfisoxazole

	Formulations					
	Synthetic		Commercial			
Coll.	Powder, mg/tab.[a]	Solution, mg/mL[b]	Tablet 1, mg/tab.[c]	Tablet 2, mg/tab.[c]	Solution, mg/mL[d]	Ointment, mg/g[e]
A	506.1	40.0	521.2	511.1	41.1	41.4
	505.1	40.2	521.7	511.8	41.5	41.2
B	505.1	40.6	511.5	501.5	42.4	41.9
	506.0	40.6	510.9	503.1	42.5	42.5
C	489.9	40.5	504.0	489.5	42.3	42.3
	490.8	40.5	499.2	487.0	42.3	42.4
D	488.3	37.8	498.7	486.2	41.1	40.9
	493.6	39.1	499.8	488.1	40.9	41.0
E	504.1	39.7	506.8	501.9	42.1	41.5
	505.4	39.7	502.0	506.6	41.8	41.3
F	498.3	40.7	508.2	503.3	41.9	40.8
	NS[f]	40.7	502.8	506.5	41.5	40.9
G	499.0	40.1	516.1	500.0	41.8	41.5
	500.3	40.3	509.5	497.5	41.7	41.9
Av.	499.4	40.0	508.0	499.6	41.8	41.5
Av. % of decl.	99.9	100.0	101.6	99.9	104.5	103.8
SD	6.690	0.792	7.660	8.730	0.513	0.579
CV, %	1.34	1.98	1.51	1.75	1.23	1.40

[a] Formulated to contain 500 mg/0.6344 g.
[b] Formulated to contain 40 mg/mL.
[c] Declared 500 mg/tab.
[d] Declared 40 mg/mL.
[e] Declared 40 mg/g.
[f] NS = not submitted.

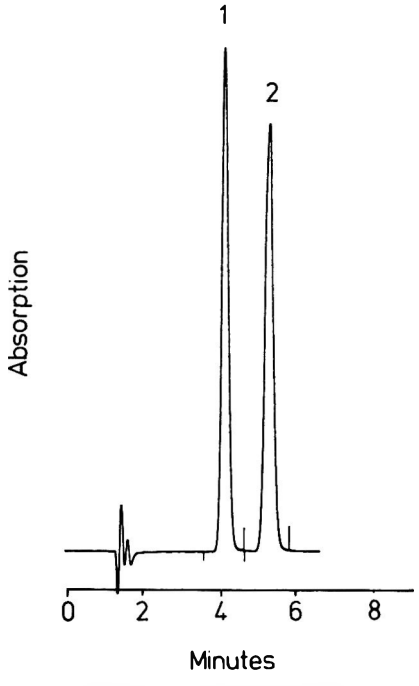

Figure 1. Chromatographic separation of 1, sulfisoxazole, and 2, sulfadimethoxime.

commercial ophthalmic ointment were shown to have coefficients of variation in the range 1.23–1.75%.

Recommendation

The selectivity of the HPLC method collaboratively evaluated in the present study is such that sulfisoxazole can be distinguished from 19 other sulfonamides having different capacity factors (1). Based on this selectivity and on the collaborative results, the Associate Referee recommends that the HPLC method proposed for the determination of sulfisoxazole in tablets, solutions, and ointments be adopted official first action.

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Joseph Lepore, Hoffmann-La Roche, Inc., Nutley, NJ

Richard Muzeni, Food and Drug Administration, Brooklyn, NY

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(1) Roos, R. W. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 851–854

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Separation and Identification of Phencyclidine and Some of Its Analogs

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1-(1-Phenylcyclohexyl)piperidine (PCP) and 9 of its analogs have been separated and identified by using thin layer chromatography, high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and infrared spectroscopy (IR). Spectral and chromatographic characteristics are discussed in terms of the structural variations observed in these compounds. Some compounds decomposed on the GC column; the HPLC procedure is a helpful substitute. A tentative scheme is proposed for the fragmentation of the major ions observed in the mass spectra. The effect of substitution on the IR spectra of the analogs was investigated.

Of all the drugs that are clandestinely synthesized, phencyclidine (1-(1-phenylcyclohexyl)-piperidine; PCP; Figure 1) has become one of the most commonly encountered by the forensic chemist. The drug, originally marketed in 1958 as an anesthetic under the trade name Sernylan® (CI-395) by Parke, Davis and Co. in the United States, was withdrawn in 1967 and it is presently controlled under Schedule II of the U.S. federal Controlled Substances Act. Since then it has been seized in various forms, capsules, tablets, white powder, etc. It is very often sprayed on parsley flakes and other vegetable matter. The relatively simple synthesis (1) may be one reason for its preponderance in the illicit drug market. However, the point of concern arises due to the reported psychic disturbances caused by the drug, which are more complicated and bizarre than for most drugs known to date and yet are least understood (2). Recently, however, the drug scene pertaining to PCP has become more complex. Law enforcement laboratories in the United States have been reporting seizures involving various analogs of PCP (U.S. Department of Justice, Drug Enforcement Administration, Washington, DC, private communication); this laboratory has also been receiving an increasing number of submissions involving the analogs of PCP.

Due to their high potential abuse and lack of accepted medical use, a number of the analogs of PCP have recently been added to the list of substances included in the Controlled Substances Act. Table 1 lists the names of these analogs and their federal schedules; structures are shown in Figure 1.

There have been some earlier reports on the identification of PCP and its analogs (3, 4). Most of the recent publications have dealt with the problem of identification and quantitation of 1-piperidinocyclohexane carbonitrile (PCC), a contaminant found in PCP, and which is known to be responsible for many of the reported fatalities associated with the drug (5–10). It has been known for some time that PCC decomposes during thin layer chromatographic (TLC) and gas chromatographic (GC) analysis (5). Lately, there have been reports which indicate that PCP also undergoes minor decomposition during GC analysis (10). However, the thienyl analogs 1-(1-(2-thienyl)cyclohexyl)piperidine (TCP), 1-(1-(2-thienyl)cyclohexyl)pyrrolidine (TCPy), and 1-(1-(2-thienyl)cyclohexyl)morpholine (TCM) all decompose considerably on the GC column (11). Thus, some of the short retention times reported earlier (3, 4) for the thienyl analogs in relation to their phenyl counterparts have been shown to be due to the formation of 1-(2-thienyl)cyclohexene as a result of thermal decomposition (11).

To circumvent the problem of decomposition under GC conditions, high performance liquid chromatography (HPLC) has been used with advantage in this investigation to separate and identify the heat-labile thienyl analogs as well as the phenyl analogs. A survey of the literature revealed no publication on the analysis of PCP analogs by using HPLC; however, a few publications (12, 13) deal with the separation of only PCP from other drugs by this technique.

Although GC–mass spectrometric (GC-MS) and infrared (IR) data on the PCP analogs studied in this manuscript have been published by other workers (3, 4), we felt that it was worthwhile to re-examine their findings to see if a comprehensive methodology could be developed for positive identification from a forensic standpoint. Since the analogs studied are structurally related, they provided a unique opportunity to

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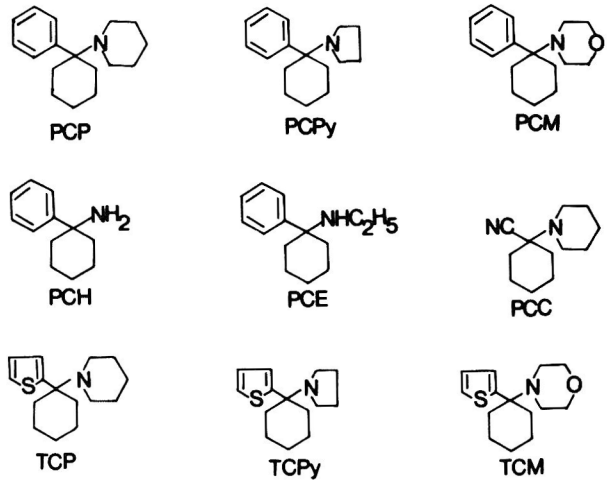

Figure 1. Structures of PCP and its analogs. See Table 1 for names of compounds.

examine the influence of structural variations on the spectroscopic and chromatographic characteristics. A tentative scheme to explain the formation of major ions in the mass spectra of the PCP analogs is proposed. The infrared spectra of the analogs were studied to find a region that could be used for quick comparison and identification of an unknown sample. Further, TLC, ultraviolet (UV), and GC techniques were also used to determine whether they could be used for preliminary screening and separation of these compounds.

METHOD

Reagents

(a) *Sample standards.*—PCP hydrochloride was obtained from the U.S. Pharmacopeial Convention, Inc., Rockville, MD 20852. All other analogs were obtained from the National Institute on Drug Abuse (NIDA), Rockville, MD. All compounds other than PCC were in hydrochloride form.

(b) *TLC solvents.*—All Spectro grade unless otherwise specified.

(c) *TLC solvent system.*—(*1*). Methanol–ammonia (100 + 1.5 v/v). (*2*). Benzene–acetone–pyridine (16 + 8 + 1 v/v/v).

(d) *TLC spray reagents.*—(*1*). Iodoplatinate reagent: Mix 0.6 mL 10% platinum chloride solution (Fisher Scientific Co.) with 150 mL aqueous KI (3.6 g). Dilute with equal volume of methanol. (*2*). Ninhydrin reagent: Prepare 0.8% (w/v) solution of ninhydrin (Baker Analyzed Reagent) in acetone. Store both spray reagents in amber bottles.

(e) *HPLC solvents.*—Methanol (HPLC grade, Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442), acetonitrile (HPLC grade, Waters Associates, Milford, MA 01757), distilled water. Filter all solvents through 0.45 μm Millipore filter.

(f) *HPLC buffer.*—0.01M 1-heptanesulfonic acid sodium salt (Eastman Kodak Co.) in combination with *N,N*-dimethyl-*N*-octylamine (0.1%

Table 1. Analogs of PCP with their abbreviations and U.S. federal schedules

Compound	Abbreviation	Federal schedule
1-(1-Phenylcyclohexyl)piperidine	PCP	II
1-(1-Phenylcyclohexyl)pyrrolidine	PCPy	I
1-(1-Phenylcyclohexyl)morpholine	PCM	—
1-Phenylcyclohexylamine	PCH	II
N-Ethyl 1-phenylcyclohexylamine	PCE	I
1-Piperidinocyclohexane carbonitrile	PCC	II
1-(1-(2-Thienyl)cyclohexyl)piperidine	TCP	I
1(1-(2-Thienyl)cyclohexyl)pyrrolidine	TCPy	—
1-(1-(2-Thienyl)cyclohexyl)morpholine	TCM	—

v/v) (ICN Pharmaceuticals Inc.) and glacial acetic acid (1% v/v) for use with Supelcosil LC-18 column, filtered through 0.45 μm Millipore filter.

(g) *HPLC mobile phase.*—27% of acetonitrile-methanol (80 + 20 v/v) solvent mixture unless otherwise specified, combined with HPLC buffer automatically in reference manifold of Model 6000A pump according to the conditions set on Model 660 solvent programmer (Waters Associates).

Apparatus

(a) *UV—visible spectrophotometer.*—Unicam SP8000 with 1 cm silica cells.

(b) *TLC plates.*—20 × 20 cm or 5 × 10 cm, coated with silica gel (E. Merck, Darmstadt, GFR).

(c) *Liquid chromatograph.*—Waters Associates, equipped with Model 660 solvent programmer, Model 6000A delivery system, Model U6K injector, Model 440 fixed wavelength (254 nm) detector connected in series with Model 450 variable wavelength detector operated at 262 or 235 nm and used in conjunction with 10 mv recorder (Houston Instrument, Austin, TX 78753); quartz flow cells each with fluid volume of 8 μL.

(d) *Prepared HPLC columns.*—150 mm × 4.6 mm id, 5 μm particle size, Supelcosil LC-18 (Supelco, Inc., Bellefonte, PA 16823).

(e) *Gas chromatograph.*—Hewlett-Packard (Avondale, PA 19311) Model 5880A equipped with glass column (60 cm × 2 mm id) packed with 2% OV-101 on 100–120 mesh Chromosorb W HP, and FID detector. Set injector and detector at 250 and 300°C, respectively; helium carrier gas at 30 mL/min.

(f) *Gas chromatograph–mass spectrometer (GC-MS).*—Varian Aerograph Series 1400 gas chromatograph interfaced with Du Pont 21-490 mass spectrometer using an all glass jet separator; ionizing voltage 70 eV, ion source 250°C, transfer lines 300°C; nickel column (180 cm × 2 mm id) packed with 3% OV-1 on 80–100 mesh Supelcoport; remaining conditions of injector and detector temperatures including helium flow rate same as in (e).

(g) *Infrared spectrometer.*—Perkin-Elmer Model PE257 grating IR. Record spectra of samples in form of KBr pellets.

Procedures

Prepare solution containing PCP (ca 1 mg/mL methanol) and each of its analogs for TLC, GC, and GC-MS studies. For HPLC, dissolve 5–7 mg/mL water. Centrifuge each sample solution before use.

TLC analysis.—Apply 5–10 μL samples on 2 separate TLC plates using a 10 μL pipet. Air-dry samples without applying heat, before placing plates in respective solvent chambers. Develop one plate in solvent system 1 and the other in solvent system 2, until solvent front reaches ca 2 cm below top edge of plates. Heat plates 10 min in 110°C oven. Spray both plates while still warm using iodoplatinate reagent. Note colors and measure R_f. Repeat TLC procedure with second set of 2 plates, and use ninhydrin spray.

HPLC analysis.—After priming pumps to remove bubbles from system, equilibrate column (ca 15 min) with mobile phase of desired composition at rate of 2 mL/min. For each analysis, use chart speed at 5 mm/min. Set variable wavelength detector at 262 or 235 nm; fixed wavelength detector is always at 254 nm. Operate HPLC column at room temperature. Inject in duplicate clear sample solutions of each analog onto column with mobile phase flow rate of 2 mL/min. Measure absorbance peak heights for each sample at 2 pairs of wavelengths (254 and 262 nm; 254 and 235 nm). Record absorbance peak ratios as (254/262 nm) and (254/235 nm), and retention times for all analogs.

Column eluate may be collected to recover pure PCP or its analogs for further characterization by GC-MS or IR.

GC and GC-MS analyses.—Separately inject 2–3 μL PCP and each of its phenyl analogs onto the nickel or glass column with temperature programming from 100 to 200°C at rate of 10°/min. Record retention time as well as mass spectrum corresponding to each peak. For thienyl analogs (TCP, TCPy, and TCM), separated on glass column, set initial oven temperature at 120°C instead of 100°C as above. To separate thienyl analogs on nickel column, inject 30–40 μL Silyl 8® with oven temperature held 5 min at 150°C before injecting sample.

Results and Discussion

UV Spectroscopy

All phenyl analogs except PCC absorb in the UV region around 251, 257, 262, and 268 nm with maximum absorbance at 262 nm in 0.1N H_2SO_4; the 3 thienyl analogs each show one broad band at 232 nm. Thus, the UV absorbance spectrum can only distinguish the phenyl from the thienyl class of compounds. The band at 232 nm for thienyl analogs is much more intense than the

phenyl absorption bands. The 20-fold increase in the extinction coefficient of thienyl compounds observed earlier (3) may be partly explained due to the interaction of the low-lying d orbitals located on the sulfur atom in the heterocyclic ring (14).

Thin Layer Chromatography

Two solvent systems were used for TLC separation of PCP and its analogs. Although TLC solvent system 1 has been used for a long time in forensic laboratories and is suitable for most nitrogenous bases (15), it has not been used in connection with PCP analogs. However, TLC solvent system 2 was reported earlier in the literature to detect contamination of PCC in PCP (5). PCC is known to eliminate a molecule of HCN to form an enamine when subjected to heat, as by applying the sample on a warm TLC plate or on the GC system. Due to its thermal instability, some of the earlier TLC studies (3, 4, 9) reported vastly different R_f values for PCC.

Table 2 lists the R_f values in the 2 solvent systems used, and the colors obtained on spraying the plates separately with 2 sprays, iodoplatinate and ninhydrin. Solvent system 1 appeared to be better overall for all analogs except PCC, which showed considerable streaking in this system. Solvent system 2, however, is best suited for PCC and also for 4 other analogs (Table 2). The ninhydrin spray seems to be a better visualizing agent, at least qualitatively; it gave spots of varying color and intensity compared with iodoplatinate spray. The different colors or shades of a given color observed while using the ninhydrin spray are particularly helpful in distinguishing PCP from 1-phenylcyclohexylamine (PCH), and TCP from TCM, since their separation in solvent system 1 is poor. It may be emphasized here that other investigators (3, 4) have reported a variety of TLC systems for the separation of phenyl and thienyl analogs.

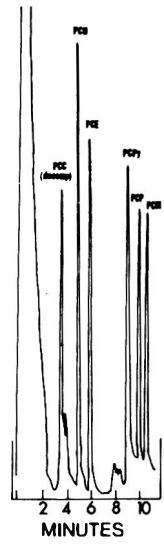

Figure 2. GC separation of PCP phenyl analogs on 3% OV-1 nickel column.

Gas Chromatography

Figure 2 shows gas chromatographic separation on a 3% OV-1 nickel column for the phenyl analogs including PCC. The retention time as well as the mass spectrum of PCC is consistent with the decomposed enamine which showed base peak at m/z 165 corresponding to its molecular ion. Unlike the phenyl analogs, their thienyl counterparts are very susceptible to thermal decomposition during GC separation (3, 11). All 3 thienyl analogs (TCP, TCPy, and TCM) decomposed completely on a nickel column irrespective of the column packing material used (3% OV-1 or 2% OV-101), but a considerable

Table 2. Thin layer chromatography of PCP and its analogs [a]

Compound	R_f System 1	R_f System 2	Color with ninhydrin	Color with iodoplatinate
PCP	0.55	0.41	grey	blue–grey
PCPy	0.31	—	dull purple	blue-grey
PCM	0.68	—	—	blue-grey
PCH	0.50	—	faint grey	bleached
PCE	0.44	—	faint grey	blue-grey
TCP	0.63	0.42	purple	blue-grey
TCPy	0.48	0.27	blue–purple	blue-grey
TCM	0.69	0.60	pink	blue-grey
PCC	0.75	0.66	deep purple	bleached

[a] Blanks indicate inconsistent R_f values due to streaking or indistinct color.

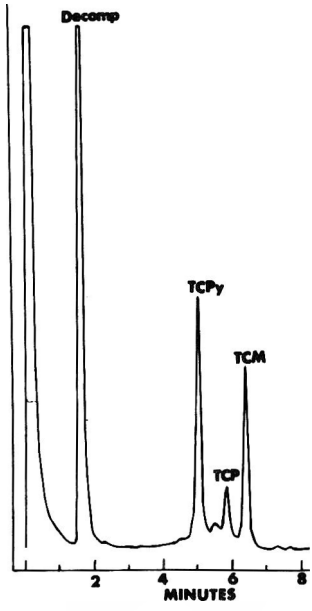

Figure 3. GC separation of PCP thienyl analogs on 2% OV-101 glass column.

amount of the undecomposed compound is detected on a glass column. This problem, however, is overcome by injecting 30–40 μL Silyl-8 onto the packed nickel column before injecting the sample. Figure 3 shows the GC separation of the thienyl analogs on the 2% OV-101 glass column. The separation of these 3 analogs, although excellent, does not permit distinguishing them from their phenyl counterparts under the same GC conditions because the retention time for each phenyl analog is longer by about 0.25 min. Similar results have been reported in the literature (3, 4, 9) where longer columns and varying phases and operating temperatures have yielded separation no better than we observed on the 2 ft glass column used in this study. In the case of the thienyl analogs, the early eluting peak at 1.6 min was identified by Legault (11) as the decomposed molecule 1-(2-thienyl) cyclohexene, and has been confirmed in this study.

Mass Spectrometry

The EI mass spectrum of PCP is shown in Figure 4. The mass spectra of the analogs studied in this manuscript have been published by other workers (3, 4, 16). A tentative fragmentation scheme (Figure 5) is proposed based on the observed mass spectrum of PCP to explain the formation of most of the major ions found. The other analogs of PCP are expected to undergo fragmentation following a similar route. While no confirmatory studies like isotope labeling were conducted to arrive at such a scheme, studying the mass spectra of a structurally related series of 1-arylcyclohexylamines such as in this investigation nevertheless provides important clues on those steps that are most likely.

Table 3 lists the 7 most prominent fragment ions found in the mass spectra of PCP and all its analogs. All these compounds show molecular ions (e.g., m/z 243 in PCP) and also an ion corresponding to (M-1) (e.g., m/z 242 in PCP) due to the loss of a hydrogen atom. The (M-1) ion can lose a neutral fragment (C_4H_8) to give rise to the ion at m/z 186 found in PCP. Further loss of a neutral C_2H_4 fragment from the ion at m/z 186

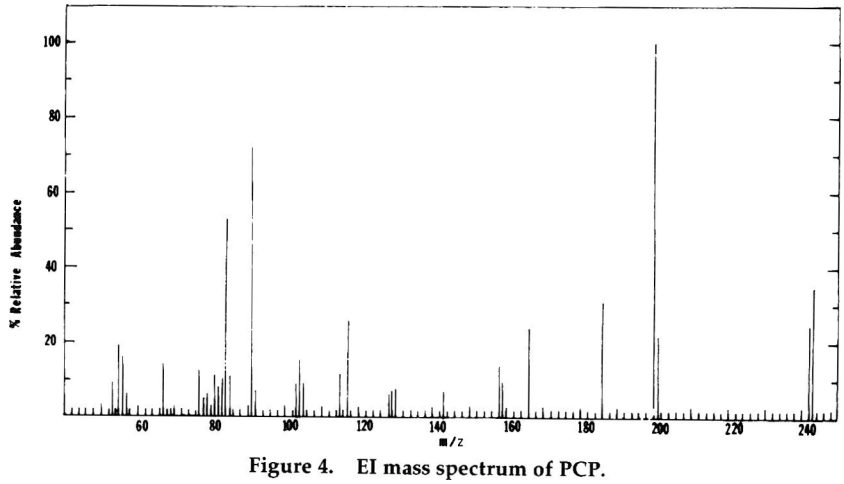

Figure 4. EI mass spectrum of PCP.

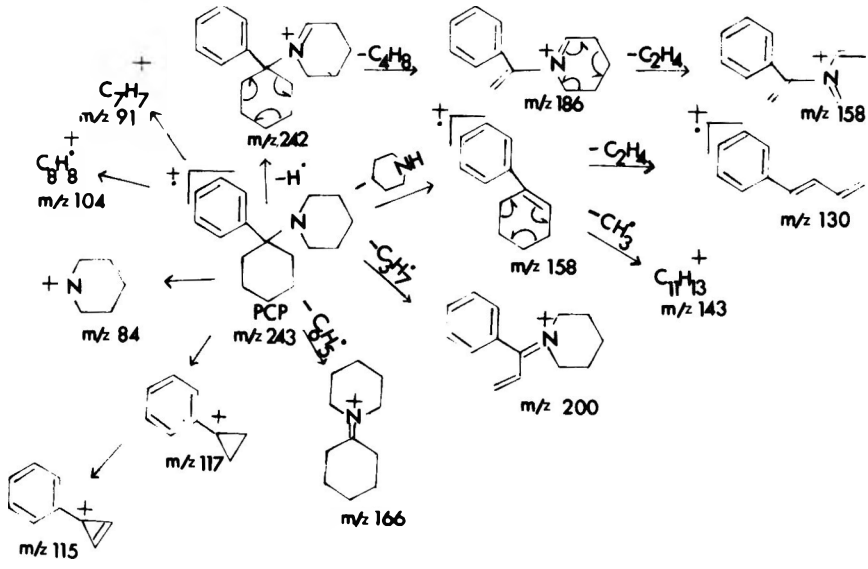

Figure 5. Tentative scheme for fragmentation of PCP.

via a retro Diels-Alder process can produce the ion at m/z 158. Alternatively, this ion at m/z 158 may be generated by the loss of piperidine from the molecular ion to form the 1-(1-phenyl)cyclohexene ion. This latter explanation appears to be more reasonable, since 2 ions at m/z 130 and 143 are observed in PCP, 1-(1-phenylcyclohexyl)pyrrolidine (PCPy), and 1-(1-phenylcyclohexyl)morpholine (PCM), and are very likely due to the loss of 28 and 15 amu from the 1-(1-phenyl)cyclohexene ion, which is in agreement with known fragmentation of cyclohexenes (17). The base peak observed at (M-43) (m/z 200 in PCP) in the PCP analogs may be assigned to the loss of a propyl radical from the molecular ion. Tentative structures may be proposed to explain the ions observed at m/z 84, 115, and 166 (Figure 5) in the mass spectrum of PCP. The ion found at m/z 91 in all the phenyl analogs due to the rupture of the benzylic bond is well known.

The fragmentation pattern for the thienyl compounds appears to follow the same pattern as for the phenyl analogs. The only difference is that TCP and TCM show a base peak at m/z 165 which seems to arise due to the $(C_{10}H_{13}S)^+$ ion, produced by losing the nitrogen-containing heterocyclic moiety rather than losing the propyl radical as observed in the phenyl series and also in TCPy (m/z 192; Table 3). The reason TCPy follows the fragmentation pattern of the phenyl analogs to produce a base peak at m/z 192 by the loss of a propyl radical is obscure.

HPLC Determination

Due to the identification difficulties associated with thermal decomposition under GC conditions, separation of PCP and its analogs has been accomplished by HPLC at room temperature on a 5 μm particle size, reverse phase Supelcosil LC-18 column using a ternary solvent system. Figure 6 gives the elution pattern of these compounds. Their identification is based on the retention time and the 2 ratios of absorbance peaks recorded at 2 pairs of wavelengths for each analog (254 and 262 nm; 254 and 235 nm) where the ratios are measured as (254/235 nm) and 254/262 nm). The selection of these wavelengths on the variable detector is due to the maximum absorbance of the phenyl and thienyl analogs around 262 and 235 nm, respectively, as mentioned under the section on UV spectroscopy. Each compound is injected in duplicate to determine the individual retention time and the 2 absorbance ratios. Then the mixture of phenyl as well as thienyl analogs is injected separately to determine the degree of resolution of each component (Figure 6).

Results are given in Table 4 under conditions of varying compositions of the mobile phase. In mobile phase 1, the paired phenyl and thienyl analogs (PCP, TCP; PCPy, TCPy; PCM, TCM) are

Table 3. Prominent ions observed in the mass spectra of PCP and its analogs

Compound	m/z (Relative abundance)					
PCP	243(33)	200(100)	186(31)	166(24)	117(26)	91(72) 84(53)
PCPy	229(27)	186(100)	172(13)	152(23)	130(13)	117(11) 91(20)
PCM	245(48)	200(100)	188(20)	168(20)	159(25)	117(24) 91(57)
PCH	175(23)	132(100)	119(14)	104(18)	91(9)	77(10)
PCE	203(28)	160(100)	146(23)	132(20)	114(21)	91(20) 72(11)
PCC	165(100)	164(60)	150(70)	138(27)	137(48)	122(31) 84(17)
TCP	249(50)	206(45)	165(100)	136(37)	123(19)	110(22) 97(91)
TCPy	235(73)	192(100)	165(61)	164(20)	120(19)	110(6) 97(46)
TCM	251(63)	208(52)	165(100)	164(38)	136(7)	123(22) 97(100)

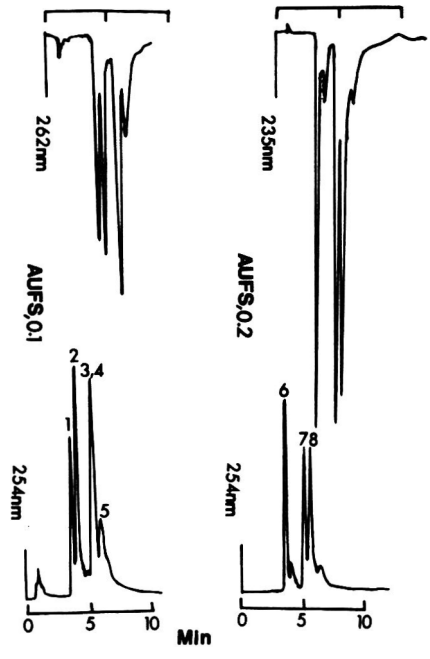

Figure 6. HPLC separation of PCP analogs on Supelcosil LC-18 column: left, phenyl analogs; right, thienyl analogs. See Table 4 for peak identification.

easily resolved at ambient temperature by HPLC, whereas under all GC conditions used in the present study as well as in the previously published reports (3, 4, 11), these paired analogs were difficult to separate. In addition, there was the problem of thermal decomposition especially of thienyl analogs. However, among all the analogs, the use of mobile phase 1 could not resolve PCPy, *N*-ethyl 1-phenylcyclohexylamine (PCE), and TCP. To separate PCPy and PCE, we developed mobile phase 2 which consists of 22% of acetonitrile-methanol mixture (80 + 20 v/v) instead of 27% as in mobile phase 1. There is no significant change in the absorbance ratios when mobile phase 2 is used. TCP can be easily differentiated from PCPy and PCE based on the values of 2 absorbance ratios (Table 4). This observation together with the fact that TCP or the other thienyl analogs are rarely seized in mixture with PCPy, PCE, or with any other phenyl analog, makes mobile phase 1 the best selection overall to achieve separation within each subgroup. Furthermore, based on the absorbance ratios alone, the phenyl analog subgroup could be easily differentiated from the thienyl analog subgroup; the values of these ratios between the 2 subgroups differ by at least a

Table 4. Absorbance peak ratios and retention distance (*R*) for PCP and its analogs separated in 2 mobile phases

No.	Compound	Mobile phase 1[b]			Mobile phase 2[c]
		R (mm)[a]	254/235 nm	254/262 nm	*R* (mm)
1	PCH	19	3.7	1.10	37
2	PCM	21	4.4	0.80	27
3	PCE	27	3.7	0.90	43
4	PCPy	28	3.8	0.76	38
5	PCP	32	4.3	0.75	43
6	TCM	16.5	0.49	12.4	—
7	TCPy	25	0.37	16.8	—
8	TCP	27.5	0.41	13.3	—

[a] Chart speed: 5 mm/min.
[b] 27% of acetonitrile–methanol mixture (80 + 20 v/v) mixed with HPLC buffer.
[c] 22% of acetonitrile–methanol (80 + 20 v/v) mixed with HPLC buffer.

factor of about 10. Thus, a set of 2 values of these absorbance ratios for each analog provides an excellent supplement to the retention times (Table 4), and the ratios could be measured easily and reproducibly. These ratios have been used in the past with advantage as one of the criteria of identification in drug analysis (18, 19) in this laboratory.

In the best knowledge of the authors, no study has appeared in the literature involving the separation of PCP analogs by HPLC. However, Trinler et al. (12) and Lurie (13) reported separation of PCP, not from its analogs, but from other drugs by using this technique. Recently, Wall and Clark reported separation of the phenyl analogs from their corresponding nitrile intermediates but only by using a reverse phase HPLC procedure (20).

It may be emphasized here that the addition of *N*,*N*-dimethyl-*N*-octylamine (0.1% v/v) to the HPLC buffer as the competing base is of paramount importance to obtain symmetrical peaks with the Supelcosil LC-18 column. In the absence of this competing base, peaks were severely tailing and the analysis of basic drugs was extremely difficult. The poor peak shapes were perhaps due to the interaction between the basic compounds and the unreacted silanol groups on the LC-18 packing material.

IR Spectroscopy

The IR spectra of the PCP analogs exhibit some very interesting features. The spectra consist of several bands arising due to the cyclohexane, the aromatic, and the heterocyclic parts of the molecule. IR spectra of all the analogs studied in this manuscript have been reported earlier (3, 16) and therefore, it was not considered necessary to reproduce the data. However, earlier reports have not discussed the observed bands in any great detail nor have they attempted to correlate the bands with structure to distinguish the analogs. Examination and comparison of the IR spectra of all the analogs reveals some interesting facts below 2000 cm^{-1}. The bands in the region 2000–4000 cm^{-1} are of limited diagnostic value because they consist of several overlapping combination bands and overtones. A weak band at 2210 cm^{-1} due to the C≡N stretch, however, does help to detect the presence of the intermediate PCC, which is often found as a contaminant in PCP.

The "finger-print" region is useful because it permits distinguishing the phenyl subgroup from the thienyl subgroup by the presence of the well known phenyl bands in the former, around 1500–1600 cm^{-1} (e.g., 1470, 1500, and 1580 cm^{-1} in PCP hydrochloride). PCH and PCE hydrochlorides exhibit the characteristic bands due to the N—H in-plane bending modes (1500 and 1580 cm^{-1} for PCH hydrochloride and 1590 cm^{-1} for PCE hydrochloride). Otherwise, the region 1000–1800 cm^{-1} is of limited use for distinguishing members within a given subgroup (PCP, PCPy, PCM; TCP, TCPy, TCM).

Bands in the IR region, 700–950 cm^{-1} particularly, are very sensitive to substitution (Figure 7). This region is useful not only to distinguish phenyl from the thienyl class, but also to differentiate within each subgroup. The majority of the bands are sharp and intense, and can be explained based on the 3 constituting moieties forming a given analog. Thus, the set of bands at 710 and 770 cm^{-1} for PCP are typical of the monosubstituted benzene molecule (e.g., 710, 775 cm^{-1} in PCPy, and 725, 780 cm^{-1} in PCM), arising due to the out-of-plane bending of the 5 vicinal hydrogens. The bands are not very

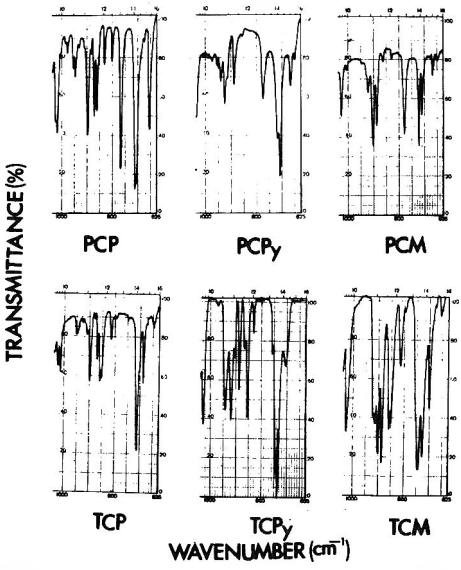

Figure 7. Comparison of IR bands observed in PCP, PCPy, PCM and TCP, TCPy, TCM.

sensitive to the nature of substitution, although the band at 725 cm^{-1} shows pronounced splitting in the case of PCM. As expected, the thienyl analogs do not exhibit this pair of bands. Instead, a strong band at 710 cm^{-1} and a band of medium intensity at 850 cm^{-1} are seen in their place in the case of TCP (e.g., 740, 850 cm^{-1} in both TCPy and TCM). Again, the absorption band at 740 cm^{-1} in TCM shows splitting as in the case of PCM. It is likely that these 2 bands in TCP, TCPy, and TCM are due to the out-of-plane bending of the free hydrogens of the 2-substituted thiophene ring. The band at 900 cm^{-1} observed both in PCP and TCP may be assigned to the in-plane rocking vibration of the methylene in the cyclohexane ring. On substitution of the piperidine ring present in PCP and TCP with pyrrolidine (e.g., in PCPy and TCPy) or with morpholine (e.g., in PCM and TCM), this band shifts to 890 cm^{-1}.

As described, the IR bands show a consistent pattern if the bands due to the cyclohexane and the phenyl moieties are accounted for and separated from the rest. This leaves the set of bands at 950 and 960 cm^{-1} in PCP which may be attributed to the CH_2 deformation of the piperidine ring.

The conclusion that these originate from the piperidine part of the molecule is substantiated by the observation that the IR spectrum of TCP also has 2 such bands at 950 and 960 cm^{-1}. Further, these 2 bands are observed at 925 and 940 cm^{-1} in PCPy and TCPy and at 900 and 930 cm^{-1} in PCM and TCM, as may be expected due to the effect of the heterocyclic ring (pyrrolidine or morpholine) on the CH_2 deformation vibration.

While these assignments in the absence of other supportive studies like deuteration or normal coordinate analysis may be best termed tentative, nevertheless, they are based on band assignments for molecules with similar structure (21-23). Although not all the peaks observed in this region (700-950 cm^{-1}) can be explained based on this study, using this region rather than any other region of IR is proposed for quick comparison and confirmation.

Conclusion

The present study describes separation and identification procedures for the most commonly encountered analogs of PCP. Identification of a given analog may be achieved by using a series of tests. However, since most of the compounds discussed are very similar in structure and many are heat labile, care must be exercised when choosing an analytical technique.

While the analogs of PCP studied are standard compounds, the data and interpretation of the results presented here may be used for the identification of forensic street samples containing any of these compounds. Most of the clandestine preparations of PCP analogs encountered in this laboratory occasionally contain a mixture of either phenyl or thienyl analogs along with the intermediate PCC. Preliminary screening of the street sample by using UV spectroscopy helps to determine whether the compound(s) belongs to phenyl or thienyl subgroup. Since PCC does not absorb in the UV region, its presence may be detected by using TLC or GC.

The thienyl analogs are susceptible to degradation while using GC, however, the early eluting peak of these analogs may well serve as an indication of the presence of TCP, TCPy, or TCM. On the other hand, HPLC has a distinct advantage over GC in that it can be used at ambient temperature thus avoiding the degradation observed under GC conditions; furthermore, HPLC can also separate the paired analogs (PCP, TCP; PCPy, TCPy; PCM, TCM), which is difficult if GC is used. These findings also make the HPLC technique suitable for quantitating the thienyl analogs. If the street sample is found to contain a mixture of the PCP analogs or is in combination

with excipients, the HPLC eluate may be collected for further confirmation by spectroscopy. Both IR and MS are specific for any given analog and at least one of these 2 techniques is recommended as a final step in the identification procedure. The low frequency IR region is sensitive to substitution.

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Rapid High Pressure Liquid Chromatographic Determination of Amitriptyline Hydrochloride in Tablets and Injectables: Collaborative Study

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A previously reported high pressure liquid chromatographic method for the determination of amitriptyline hydrochloride in dosage forms was modified to permit its use as a stability-indicating method. The modified method, entailing a nitrile bonded microparticulate column, a methanol-0.005M ammonium acetate (90 + 10) mobile phase, and photometric detection at 239 nm, was collaboratively tested by 10 laboratories. Each collaborator received samples of synthetic and commercial tablets and injections. The recovery from a synthetic injection at the 10.06 mg/mL spiking level averaged 98.6%. The amount of declared found in commercial injections averaged 103.1%. The pooled reproducibility SD (CV%) and repeatability SD (CV%) were ± 2.12 (2.15) and ± 1.81 (1.84), respectively. The recovery from synthetic tablet composite at the 7.45% spiking level averaged 102.0%. The amount of declared found for commercial 25 mg and 100 mg tablets averaged 96.7 and 97.9%, respectively. The pooled reproducibility SD (CV%) and repeatability SD (CV%) for these 3 tablet samples were ± 1.89 (1.86) and ± 1.66 (1.64), respectively. Content uniformity analysis of commercial 25 mg and 100 mg tablets (n = 10) gave amounts of declared values averaging 100.5% (range 92.4-108.8%) and 99.3% (range 89.6-107.0%), respectively. The pooled reproducibility SD (CV%) and repeatability SD (CV%) were ± 3.23 (3.2) and ± 2.78 (2.8), respectively. A commercial injectable preparation spiked with dibenzosuberone was also collaboratively analyzed by a thin layer method. The method was adopted interim official first action.

Amitriptyline hydrochloride is an antidepressant commercially available in tablet and injectable forms. The compendial assay of the drug substance entails a nonaqueous titration (1), whereas that of the dosage forms requires a preliminary oxidative step to UV-absorbing molecules followed by spectrophotometry (2). The compendial identification test for amitriptyline hydrochloride (1), based on the determination of its UV absorption spectrum and calculation of the absorptivity value at the wavelength of maximum absorbance, does not distinguish it from products arising from the degradation of its aliphatic side chain.

According to Enever et al. (3), the autoclaving of an aqueous solution of amitriptyline hydrochloride at 115–116°C in the presence of excess oxygen results in the formation of 3 degradation products identified as 3-(propa-1,3-dienyl)-1,2:4,5-dibenzocyclohepta-1,4-diene, dibenzosuberone, and 3-(2-oxoethylidene)-1,2:4,5-dibenzocyclohepta-1,4-diene. Henwood (4) reported the autooxidation of amitriptyline hydrochloride to dibenzosuberone after standing at room temperature. In this laboratory these 3 degradation products were detected by thin layer chromatography (TLC) in a commercial injection that was exposed to air at ambient temperature for as little as 2 h.

Butterfield and Sears (5) described a high pressure liquid chromatographic (HPLC) method for the simultaneous determination of amitriptyline hydrochloride and perphenazine in 2-component tablet formulations. We have introduced minor modifications into this method to improve its sensitivity and to provide a means of assaying amitriptyline hydrochloride in the presence of dibenzosuberone. The modified HPLC method has been subjected to intra- and interlaboratory collaborative studies, the results of which are the subject of this report.

Collaborative Study

Each of 10 collaborators received 9 samples, labeled A to H, 5 of which were to be examined for amitriptyline hydrochloride, 2 for content uniformity, and 2 for purity and identification tests. Samples A and B represented a synthetic

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solution for injection (10.06 mg/mL) and a commercial solution for injection (10.00 mg/mL), respectively. Samples D and E were commercial 25 mg and 100 mg tablets, respectively. Samples F and G were the corresponding powdered composite samples of D and E, respectively. Sample H was an authentic mixture representing a typical commercial tablet. Sample C was reserved for TLC and infrared (IR) spectroscopic tests, and sample I only for a TLC test. All samples except C and I required HPLC assays in duplicate. Samples B, F, and G required TLC, IR, and the determination of retention times to serve as identification tests.

The collaborators were also sent an instruction sheet, a copy of the method, vials containing standards, and reporting forms. For the analysis of composite tablet samples, each collaborator was informed of the average tablet weights and label declarations, and instructed to use a sample weight equivalent to 10 mg of amitriptyline hydrochloride.

Amitriptyline in Tablets and Injectables
Liquid Chromatographic Method
Interim Official First Action

37.D01 *Principle*

Amitriptyline content of tablets and injectables is detd by liq. chromatography, using trifluoperazine as internal std and UV detection at 239 nm.

37.D02 *Apparatus and Reagents*

(a) *Liquid chromatograph.*—Tracor Model 950 solvent pump, Model 970A variable wavelength detector, Model 26325 recorder (Tracor Instruments Inc., Austin, TX 78721), and 20 μL Rheodyne Model 7120 loop injector (Rheodyne Inc., Berkeley, CA 94710). *Operating conditions:* column temp., ambient; solv. flow rate, 1.33 mL/min; detector wavelength, 239 nm; attenuation, 16 AUFS; recorder, 1 mV; chart speed, 1 in./4 min.

(b) *Chromatographic column.*—Stainless steel, 300 × 3.9 mm id, packed with 10 μm μBondapak CN (Waters Associates, Inc.), or equiv.

(c) *Methanol.*—AR grade (Fisher Scientific Co.).

(d) *Mobile phase.*—MeOH–0.005M ammonium acetate (90 + 10).

(e) *Culture tubes.*—95 × 25 mm with screw cap (Kimble).

(f) *Internal std soln.*—Accurately prepare ca 0.5 mg Trifluoperazine HCl Ref. Std/mL MeOH.

(g) *Std soln.*—0.04 mg/mL. Accurately weigh ca 10 mg USP Amitriptyline HCl Ref. Std and transfer to 250 mL vol. flask. Dissolve in 1 mL MeOH, add 25.0 mL internal std soln, dil. with MeOH, and mix.

37.D03 *Sample Preparation*

(a) *Tablets.*—Weigh and finely powder ≥20 tablets. Accurately weigh and transfer amt of powd. equiv. to 10 mg amitriptyline HCl into screw-cap culture tube and add 25.0 mL internal std soln. Tumble on rotator 15 min at ca 30 rpm, and filter, if necessary. Dil. accurately measured vol. of soln with MeOH to ca 0.04 mg/mL.

(b) *Single tablet.*—Place one tablet in 95 × 25 mm screw-cap culture tube and crush to fine powd. with glass rod. Add 25.0 mL MeOH and mix. Tumble on rotator 15 min at ca 30 rpm, and filter, if necessary. Pipet accurately measured aliquot (A) of this soln, equiv. to 2 mg amitriptyline HCl, into 50 mL vol. flask, add 5.0 mL internal std soln, dil. to vol. with MeOH, and mix.

(c) *Injectables.*—Accurately pipet vol. of injectable, equiv. to 10 mg amitriptyline HCl, into 50 mL vol. flask, add 25.0 mL internal std soln, dil. to vol. with MeOH, and mix. Dil. accurately measured vol. of this soln with MeOH to ca 0.04 mg/mL.

37.D04 *Determination*

Equilibrate system with mobile phase at 1.33 mL/min, until baseline is steady. Inject measured vol. of std soln into chromatograph by microsyringe or sampling valve. Adjust injection vol. and operating parameters so amitriptyline HCl in std soln injection gives peak ht ca 60% full scale and retention time ca 7 min. Under these conditions, 5 replicate injections of std soln should give coefficient of variation of ≤3% and resolution factor (R) between the 2 main peaks should be ≥1. Make alternate injections of equal vols. of sample and std solns. Measure peak hts for amitriptyline HCl and internal std in sample and std solns, and det. response ratios.

37.D05 *Calculations*

Tablets: $\text{mg/tablet} = RR/RR' \times C \times T/W$

Single tablet: $\text{mg/tablet} = RR/RR' \times C \times 5/A$

Injectables: $\text{mg/mL} = RR/RR' \times C/V$

where RR and RR' = ratio of amitriptyline HCl

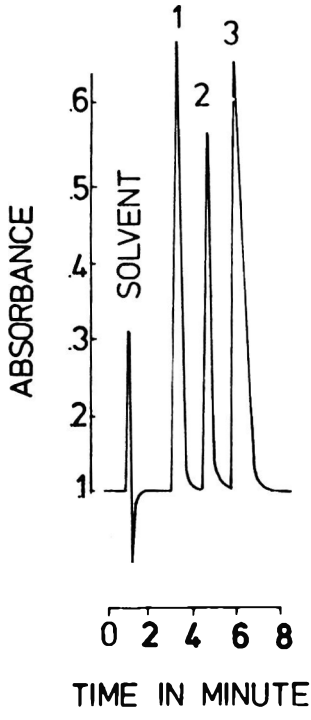

Figure 1. HPLC separation of 1, dibenzosuberone; 3, amitriptyline hydrochloride; and 2, trifluoperazine hydrochloride (internal standard) on Microbondapak CN. Mobile phase, methanol-0.005M ammonium acetate (90 + 10); flow rate, 1.33 mL/min; wavelength, 239 nm, 16 AUFS.

peak ht to internal std peak ht for sample and std solns, resp.; C = mg amitriptyline HCl in 250 mL std soln; T = av. tablet wt, g; W = wt sample taken, g; A = aliquot taken, mL; V = vol. injectable taken, mL.

Results and Discussion

An evaluation of published HPLC methods for the determination of amitriptyline hydrochloride in biological samples (6, 7) and pharmaceuticals (5, 8, 9) indicated that the method of Butterfield and Sears (5) was the method of choice for regulatory work because of its simplicity and the possibility of using readily available chemicals. By altering the ratio of the components of the mobile phase and setting the detector to 239 nm, this method was made more sensitive and suited for stability-indicating testing of dibenzosuberone in amitriptyline hydrochloride samples. With this modified HPLC method, amitriptyline hydrochloride could be separated from dibenzosuberone, its major degradation product, and from trifluoperazine hydrochloride, the internal standard, in less than 7 min (Figure 1).

Table 1. Recovery of amitriptyline hydrochloride added to commercial tablets and injections by HPLC method

Mfr	Added, mg	Found, mg	Rec., %
A[a]	1.19	1.21	101.7
B	1.19	1.19	100.0
C	1.19	1.18	99.2
D	1.19	1.20	100.8
E	1.19	1.19	100.0
Pooled av. rec., %			100.3

[a] Injection.

Although dibenzosuberone is the only degradation product reported to be spontaneously formed from amitriptyline base upon oxidation at room temperature (4), in the course of this study we found that the other 2 degradation products, namely, 3-(propa-1,3-dienyl)-1,2:4,5-dibenzocyclohepta-1,4-diene and 3-(2-oxoethylidene) - 1,2:4,5 - dibenzocyclohepta - 1,4-diene, may also be formed in aqueous solutions under the same experimental conditions. Exposure of a commercial aqueous solution of amitriptyline hydrochloride to air at room temperature for up to 2 h resulted in a sample which, when separated by TLC with the chromatographic conditions described by Zingales (10), produced 3 additional spots. The major spot was identified as dibenzosuberone by comparison with an authentic sample. The other 2 degradates were subjected to chromatography and cochromatography with an aqueous solution of amitriptyline hydrochloride degraded according to the method of Enever et al. (3), and all 3 degradation products were tentatively identified. These results clearly established the critical role of air in promoting the self-degradation of amitriptyline and lend support to the findings of Roman et al. (8) who were unable to detect any evidence of decomposition when amitriptyline hydrochloride injections with a low ratio of head space oxygen to drug were stored either at 80°C for up to 3 months or at room temperature for longer than 5 years; similar results were obtained by Enever et al. (11) who stored the drug in sealed ampules under nitrogen.

In agreement with Butterfield and Sears (5), methanol was found superior to water, water-methanol, or water-acetonitrile mixtures, for the extraction of amitriptyline hydrochloride from tablets, because the aqueous extractants invariably resulted in the formation of troublesome suspensions. The reproducibility of the extrac-

Table 2. Results of analyses of amitriptyline hydrochloride dosage forms by HPLC and USP XX methods

Mfr	Labeled, mg	Individual tablet assay				Composite sample assay			
		Found, mg (ITA)		Found, %		Found, mg		Found, %	
		USP	HPLC	USP	HPLC	USP	HPLC	USP	HPLC
A	10	9.41	9.36	94.10	93.6	9.40	9.21	94.0	92.1
A	150	150.70	148.70	100.40	99.1	148.60	147.83	99.1	98.6
B	10	9.62	9.93	96.20	99.3	9.87	9.80	98.7	98.0
B	150	152.40	145.70	101.60	95.0	151.00	148.39	100.7	99.0
C	10	9.64	9.76	96.4	97.6	9.79	9.70	97.9	97.0
C	100	100.20	102.40	100.20	102.4	101.50	100.50	101.5	100.5
D	10	10.59	9.97	106.00	99.7	10.35	10.05	103.5	100.5
D	100	104.20	104.10	104.20	104.1	104.20	102.50	104.2	102.5
E	25	26.50	25.90	105.90	103.5	25.70	25.29	102.8	101.3
E	150	157.20	156.00	104.80	104.0	153.70	157.02	102.5	104.7
A[a]	10	—	—	—	—	10.30	10.39	103.0	103.9

[a] Injection.

tion procedure was evaluated by tumbling crushed individual tablets with 25 mL of internal standard solution for 5, 10, and 15 min, respectively. In each case, the suspension was centrifuged briefly, and 1 mL of the supernatant was diluted accordingly and analyzed chromatographically. From measurements of peak height ratios, reproducible extractions were obtained within 5 min.

Before the submission of the HPLC method to collaborative evaluation, intralaboratory studies were conducted to determine its precision and accuracy. Samples of 4 commercial tablets and 1 commercial injection were spiked with the same amount of amitriptyline hydrochloride and then assayed by the HPLC method. Table 1 shows that the average recoveries for tablets and injectables were 100.0 and 101.7%, respectively. Table 2 compares the assay values obtained by the proposed and compendial methods for individual tablet samples and their corresponding composites. Among individual tablet assays, differences in values between methods ranged from 0.05 to 6.17% of declared. For all other samples tested, differences were less than 2% of declared. The data on content uniformity testing presented in Table 3 indicated good agreement between methods, with differences generally within less than 2% of each other. A recovery study from synthetic samples simulating tablets and injectables gave the results shown in Table 4. For 6 replicate assays each of synthetic tablets and injectables, the recovery values averaged 99.7 and 100.4%, respectively.

The collaborative results for the recovery of amitriptyline hydrochloride from a synthetic injectable formulation are given in Table 5. The average recovery value at the 10.06 mg/mL spiking level was 98.6 ± 1.22%, with a coefficient of variation (CV) of 1.24%. A commercial injectable was found to contain an average of 103.1 ± 2.04% with a CV of 1.98% (Table 5). According to Dixon's test (12) all laboratories were within range. However, the modified Cochran test (12)

Table 3. Comparison of results for content uniformity testing by HPLC and compendial methods

Mfr	Label, mg	Range found, %		Av. found, %		CV, %	
		USP	HPLC	USP	HPLC	USP	HPLC
A	10	90.9–100.1	88.1– 96.0	94.1	93.6	3.4	2.9
A	150	99.7–102.8	96.0–101.9	100.4	99.1	1.4	2.3
B	10	90.7– 99.7	93.8–102.9	96.2	99.3	2.8	2.6
B	150	99.1–105.4	86.7– 99.7	101.6	95.0	2.2	4.2
C	10	91.4– 98.8	91.8–101.9	96.4	97.6	2.4	2.8
C	100	95.5–116.9	97.1–107.9	100.1	102.4	3.8	2.8
D	10	103.1–110.0	93.0–103.9	105.9	99.7	2.2	3.3
D	100	99.7–109.4	100.7–106.7	104.2	104.1	3.2	1.9
E	25	102.9–110.5	101.6–105.4	105.9	103.5	1.9	1.2
E	150	102.1–106.7	102.9–104.9	104.8	104.0	1.3	0.5

Table 4. Recovery of amitriptyline hydrochloride from synthetic tablet and injection formulations by HPLC method

Amitriptyline	Tablet	Injection
Added	10 mg/134.16 g	10.06 mg/mL
Av. found ± SD,[a] %	100.4 ± 0.914	99.7 ± 0.303
Range found, %	99.7 –102.0	99.3–100.1
CV, %	0.911	0.304

[a] $n = 6$.

found one collaborator reporting outlying results for the commercial injectable sample (Table 5). A one-way analysis of variance (ANOVA) test showed that neither the between-collaborators F-statistic nor the interaction F-statistic was significant at the 95% confidence limit ($F <$ 0.05).

The collaborative results for synthetic and commercial tablet samples are given in Table 6. The recovery value at 7.45% spiking level was 102.0 ± 0.127% with a CV of 1.67%. One set of results was found to be an outlier by the modified Cochran's test. The average assay value for 100 mg tablets was 97.9% of declared with one set of results found to be an outlier by Dixon's test and another one by the modified Cochran's test. The average assay value for the 25 mg tablets was 96.7% of declared, with no collaborator reporting outliers. The combined reproducibility SD and repeatability SD values for all 3 samples were ± 1.89 and ± 1.66, respectively. The corresponding CV values were 1.86 and 1.64%, respectively. Good agreement was noted between the intralaboratory and interlaboratory results. The ANOVA test showed that the between-collaborators F-statistic was significant at the 95% confidence level ($F < 0.05$), but the interaction F-statistic was not ($F < 0.05$). However, since the precision of the method was less than 2%, the variance due to collaborators was not large enough to affect the precision of the method significantly.

Collaborative results for the content uniformity testing of commercial tablets are presented in Tables 7 and 8. The average values found for 100 mg and 25 mg tablets were 99.3 and 100.5% of declared, respectively. The pooled reproducibility SD and repeatability SD were ± 3.23 and ± 2.78, respectively, with corresponding CV values of 3.2 and 2.8%, respectively. No outliers were found by Thompson's test (12).

Statistical evaluations made in this study exclude all those results found as outliers. No difficulties with the HPLC method were reported by any of the collaborators and no procedural modifications were suggested.

Table 5. Collaborative results for determination of amitriptyline hydrochloride in synthetic and commercial injectables by HPLC method

	Sample A[a] found, %			Sample B[b] found, %		
Coll.	Run 1	Run 2	Av.	Run 1	Run 2	Av.
1	98.6	98.9	98.8	101.9	102.0	102.0
2	98.7	99.6	99.2	102.9	102.3	102.6
3	97.1	97.0	97.1	102.0	102.0	102.0
4	98.3	98.6	98.5	101.8	101.7	101.8
5	100.2	100.2	100.2	104.7	104.1	104.4
6	99.0	99.7	99.4	102.0	104.0	103.0
7	100.0	100.1	100.1	107.5[c]	108.1[c]	107.8[c]
8	98.7	97.6	98.2	101.4	101.5	101.5
9	98.4	97.7	98.1	101.3	101.3	101.3
10	96.4	96.4	96.4	104.9	105.0	105.0
Av., %			98.6			103.1
SD			1.22			2.04
CV, %			1.24			1.98
Reproducibility SD				2.12		
Reproducibility CV, %				2.15		
Repeatability SD				1.81		
Repeatability CV, %				1.84		

[a] Synthetic formulation spiked with amitriptyline hydrochloride at 10.06 mg/mL level.
[b] Commercial sample with declared value of 10.0 mg/mL.
[c] Outlier by modified Dixon's test (12).

Table 6. Collaborative results for determination of amitriptyline hydrochloride in synthetic and commercial tablets by HPLC method

	Synthetic tablet[a] rec., %			25 mg tablet found, %			100 mg tablet found, %		
Coll.	Run 1	Run 2	Av.	Run 1	Run 2	Av.	Run 1	Run 2	Av.
1	99.9	100.0	100.0	94.4	96.4	95.4	96.4	96.8	96.6
2	100.9	101.5	101.2	94.4	94.4	94.4	95.4	99.5	97.5[b]
3	102.0	102.8	102.4	95.7	95.2	95.5	97.9	97.9	97.9
4	102.3	103.7	103.0	96.6	94.8	95.7	98.0	99.2	98.6
5	100.0	100.0	100.0	95.3	95.2	95.3	97.3	97.3	97.3
6	102.3	103.8	103.1	101.2	102.0	101.6	102.1[c]	103.3[c]	102.7[c]
7	101.6	100.9	101.3	97.2	96.9	97.1	97.7	98.2	98.0
8	102.8	100.1	101.5	96.8	96.8	96.8	97.9	97.7	97.8
9	100.0	103.1	101.6	95.8	96.0	95.9	97.5	97.5	97.5
10	105.5[b]	106.1[b]	105.8[b]	99.2	99.2	99.2	100.1	99.7	99.9
Av., %			101.5			96.7			97.9
SD			1.13			2.17			1.75
CV, %			1.11			2.25			1.78
Reproducibility SD				1.89					
Reproducibility CV, %				1.86					
Repeatability SD				1.64					

[a] Spiked at the 7.45% level.
[b] Outlier by modified Dixon's test (12).
[c] Outlier by Dixon test (12).

Table 7. Collaborative results for determination of amitriptyline hydrochloride in individual 25 mg commercial tablets by HPLC method

	Found, % of declared									
Tablet	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6	Coll. 7	Coll. 8	Coll. 9	Coll. 10
1	98.0	102.3	96.7	100.9	100.4	105.0	105.8	102.4	98.8	101.8
2	101.2	104.2	102.8	103.5	100.2	107.0	93.7	102.8	100.4	100.8
3	100.8	99.2	92.9	102.4	100.4	108.7	104.9	98.8	100.0	102.0
4	92.4	96.0	105.4	102.0	98.1	97.4	103.4	99.6	96.0	104.0
5	100.0	97.6	101.6	104.4	99.2	102.0	99.9	94.8	99.2	99.4
6	98.4	99.6	100.2	99.6	103.0	97.4	108.8	100.4	100.0	95.4
7	99.2	103.3	93.4	100.5	102.3	100.7	96.9	99.6	100.8	101.6
8	98.8	99.8	96.5	104.6	101.6	104.9	102.9	100.0	100.8	101.7
9	98.4	103.4	95.9	100.0	101.0	104.7	102.4	100.0	99.6	94.7
10	96.8	96.0	98.4	105.1	97.0	105.2	101.4	102.4	101.2	99.3
Av.	98.4	100.1	98.4	102.3	100.3	103.3	102.0	100.1	99.7	100.1
Low range	92.4	96.0	92.9	99.6	97.0	97.4	93.7	94.8	96.0	94.7
High range	101.2	104.2	105.4	104.6	103.0	108.7	108.8	102.8	101.2	104.0

Table 8. Collaborative results for determination of amitriptyline hydrochloride in individual 100 mg commercial tablets by HPLC method

	Found, % of declared									
Tablet	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6	Coll. 7	Coll. 8	Coll. 9	Coll. 10
1	99.3	95.0	100.4	98.3	97.8	104.4	96.6	101.5	101.0	100.3
2	96.0	89.6	102.0	96.6	100.7	103.0	96.7	93.4	99.1	104.8
3	94.6	91.9	103.8	103.8	101.1	99.7	97.9	95.6	94.3	104.4
4	93.9	97.7	101.1	103.7	98.4	99.6	101.4	97.2	98.1	99.0
5	100.2	92.3	99.8	98.3	102.6	97.4	98.1	100.3	98.6	99.2
6	102.2	97.6	101.1	98.3	99.4	100.0	102.3	99.3	94.5	99.4
7	100.8	100.9	100.6	103.1	99.0	105.1	101.8	98.4	97.4	99.1
8	99.2	96.7	101.5	100.6	101.3	107.0	97.0	98.6	96.7	99.2
9	94.0	97.7	100.9	99.2	104.5	100.2	100.7	96.7	97.4	100.3
10	95.8	95.2	100.7	100.8	103.8	106.8	98.3	96.2	98.5	99.7
Av.	97.6	95.5	101.2	100.3	100.9	102.3	99.1	97.7	97.6	100.5
Low range	93.9	89.6	99.8	96.6	97.8	97.4	96.6	93.4	94.3	99.0
High range	102.2	100.9	103.8	103.8	104.5	106.9	102.3	101.5	101.0	104.8

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Atomic Absorption Spectrophotometric Determination of Mercury in Mercury-Containing Drugs: Collaborative Study

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An atomic absorption spectrophotometric (AAS) method applicable to a wide variety of mercury-containing drugs has been developed and subjected to a collaborative study. Samples were digested with a water-HCl-HNO_3 (4 + 3 + 1) mixture, and the mercury was determined in solution by AAS. High levels of mercury were measured with a conventional air-acetylene flame, whereas low levels were measured by the flameless technique. Each of 7 collaborators received duplicate synthetic samples of a tincture, an ophthalmic solution, and an antiseptic solution, and duplicate commercial samples of an ointment and an injectable. The overall mean value found by collaborators for mercury in these samples was 100.13%. The corresponding overall repeatability SD (CV, %) and reproducibility SD (CV, %) values were 2.18 (2.18) and 3.38 (3.38), respectively. The proposed AAS method has been adopted official first action.

The methods for analysis of mercury-containing drugs appearing in the AOAC chapter on General Drugs (1) and dating as far back as 1927 are generally nonspecific. The determinative step consists of either a gravimetric precipitation of mercury as mercuric sulfide or an indirect iodometric titration (2). A more modern method using vapor-phase atomic absorption has been recently published (3). The development of a general method for analysis of mercury-containing drugs is complicated by the existence of a large variety of drug preparations and by the wide variation in their mercury concentrations, which range from parts per million to percent levels. AAS offers the needed flexibility together with specificity and adequate sensitivity. The use of a conventional air-acetylene flame permits the determination of high levels of mercury, whereas low levels can be measured by the flameless technique.

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Mercury in Mercury-Containing Drugs

Atomic Absorption Spectrophotometric Method

Official First Action

36.D01 ***Principle***

Samples are digested in H_2O-HCl-HNO_3, and Hg is detd by AAS using air-C_2H_2 flame or flameless technic (low Hg levels).

36.D02 ***Apparatus***

Rinse all glassware before use with HNO_3 (1 + 1) followed by H_2O. For low Hg levels, decontaminate boiling flasks before use as follows: Add 5 mL H_2O-HCl-HNO_3 (4 + 3 + 1), place on steam bath 20 min, and rinse with H_2O.

(**a**) *Atomic absorption spectrophotometer.*—Equipped with air-C_2H_2 flame, or equipped with Hg hollow cathode lamp and gas flow-thru cell (Fig. **25:02**), 25(id) × 115 mm with quartz windows cemented in place. Operating conditions: wavelength 253.7 nm, slit width 160 μm, lamp current 3 mA, and sensitivity scale 2.5.

(**b**) *Diaphragm pump.*—Neptune Dyna-Pump, or equiv. Coat diaphragm and internal parts of pump with acrylic-type plastic spray. Use 16 gage Teflon tubing for all connections.

(**c**) *Gas inlet adapter.*—24/40 ⅋ (Kontes Glass Co. No. K-181000).

(**d**) *Digestion flask.*—250 mL flat-bottom boiling flask with 24/40 ⅋ joint.

36.D03 ***Reagents***

(**a**) *Reducing soln.*—Mix 50 mL H_2SO_4 with ca 300 mL H_2O. Cool to room temp. and dissolve 15 g NaCl, 15 g hydroxylamine sulfate, and 25 g $SnCl_2$ in soln. Dil. to 500 mL.

(**b**) *Diluting soln.*—To 1 L vol. flask contg 300–500 mL H_2O, add 58 mL HNO_3 and 67 mL H_2SO_4. Dil. to vol. with H_2O.

(**c**) *Magnesium perchlorate.*—Drying agent placed in filter flask (Fig. **25:02**). Replace as

needed. (*Caution:* $Mg(ClO_4)_2$ is explosive when in contact with org. substances.)

(**d**) *Mercury stock soln.*—1000 μg/mL. Dissolve 0.1354 g $HgCl_2$ in 100.0 mL H_2O.

(**e**) *Digestion soln.*—H_2O-HCl-HNO_3 (4 + 3 + 1). Prepare immediately before use.

(**f**) $K_2Cr_2O_7$ *soln.*—5%, aq.

36.D04 *Sample Preparation*

(**a**) *Ointments.*—Mix sample thoroly and accurately weigh portion contg ca 5 mg Hg into 50 mL beaker. Add 5 mL H_2O-HCl-HNO_3 (4 + 3 + 1). Cover with watch glass and heat on steam bath 30 min. Cool to room temp., swirl beaker to coagulate fat, and decant soln and three 10 mL H_2O rinses into 50 mL vol. flask. Add 2 mL 5% $K_2Cr_2O_7$, dil. to vol., and mix. Prep. reagent blank, beginning "Add 5 mL H_2O-HCl-HNO_3 . . .".

(**b**) *Tinctures.*—Pipet aliquot contg ca 5 mg Hg into 50 mL vol. flask, place on steam bath, and evap. almost to dryness in current of air. Add 5 mL H_2O-HCl-HNO_3 (4 + 3 + 1), and heat on steam bath 30 min. Blow air into flask 2–3 min, while swirling contents, to expel N oxides. Cool to room temp., add ca 30 mL H_2O and 2 mL $K_2Cr_2O_7$ soln, dil. to vol. with H_2O, and mix. Prep. reagent blank, beginning "Add 5 mL H_2O-HCl-HNO_3 . . .".

(**c**) *Injectables.*—Pipet aliquot contg ca 5 mg Hg into 50 mL vol. flask, add 5 mL H_2O-HCl-HNO_3 (4 + 3 + 1), and proceed as in (**b**), beginning ". . . and heat on steam bath . . .".

(**d**) *Preservatives and solns (or samples contg low levels of Hg).*—Pipet duplicate aliquots contg 0.5 μg Hg (0.1 mL Eppendorf pipet, or equiv., dilg sample if necessary), into sep. decontaminated 250 mL boiling flasks, add 5 mL H_2O-HCl-HNO_3 (4 + 3 + 1) to each flask, and heat on steam bath 30 min. Cool to room temp., and add 95 mL dilg soln, (**b**). Prep. 2 reagent blanks, beginning "Add 5 mL H_2O-HCl-HNO_3 . . .".

36.D05 *Standard Preparation*

(**a**) *0 and 100 μg Hg/mL std solns (for samples a, b, and c).*—Pipet 0 and 5 mL 1000 μg/mL Hg stock soln into 50 mL vol. flasks, add 5 mL H_2O-HCl-HNO_3 (4 + 3 + 1), ca 30 mL H_2O, and 2 mL $K_2Cr_2O_7$ soln, dil. to vol. with H_2O, and mix.

(**b**) *0.5 μg Hg std soln (for sample d).*—Dilute 1000 μg/mL Hg stock soln to 5 μg/mL. Pipet duplicate 0.1 mL aliquots of this soln (Eppendorf pipet or equiv.) into sep. decontaminated 250 mL boiling flasks, (**d**). Add 5 mL H_2O-HCl-HNO_3 (4 + 3 + 1), and heat on steam bath 30 min. Cool to room temp., add 95 mL dilg solution, (**b**), and mix.

36.D06 *Determination*

(**a**) *Samples a, b, and c.*—Operate atomic absorption spectrophtr with air-C_2H_2 flame according to manufacturer's specifications. Zero instrument with 0 μg/mL Hg std soln, and measure A of 100 μg/mL Hg std soln, blank soln, and sample solns, using 4× scale expansion.

(**b**) *Sample d.*—Adjust output of pump to ca 2 L air/min by regulating speed of pump with variable transformer. Connect app. as in Fig. **25:02**, except for gas inlet adapter. With pump working and spectrophr zeroed, add 20 mL reducing soln to dild aliquot. Immediately connect gas inlet adapter and aerate ca 3 min. (Adjust aeration time to obtain max. A.) Record A, disconnect pressure on "out" side of pump, and open vent on filter flask to flush system. Analyze in following sequence: reagent blank, 0.5 μg Hg std soln, sample solns, and 0.5 μg/mL std soln.

36.D07 *Calculations*

(**a**) Flame AAS:

$$\text{mg Hg/g or mL} = (A - A_B) \times C'/(A' \times W \times 20)$$

(**b**) Flameless AAS:

$$\text{mg Hg/g or mL} = (A - A_B)/(A' - A_B) \times (C'/V) \times F \times 1/1000$$

where A, A_B, and A' = absorbance of sample, blank, and std solns, resp.; C' = concn of std soln (μg/mL, flame AAS; μg, flameless AAS); W = wt (g) or vol. (mL) of sample taken; V = vol. sample (mL) added to 250 mL boiling flask; F = diln factor if sample was dild.

Collaborative Study

Seven collaborators from 7 different laboratories received duplicate commercial samples of an ointment and an injectable and duplicate synthetic samples of a tincture, an ophthalmic solution, and an antiseptic solution. Samples were randomly numbered from 1 to 10 with no indication of the presence of duplicates. Collaborators also received detailed information and instructions for the AAS method, and were asked to submit their results and worksheets for the proposed method to the Associate Referee.

Table 1. In-house evaluation of proposed AAS method for precision and accuracy

Sample	Found, %	Added, %	Total found, %	Rec., %
Commercial:				
Yellow mercuric oxide ophthalmic ointment, 1%	1.028[a]	2.183	3.272	102.8
Thimerosal soln, NF, 0.1%	0.104[b]	—	—	—
Thimerosal eye drops, 0.001%	0.000	0.0124	0.0125	100.8
Synthetic:				
Mercurous chloride (calomel) ointment, 6.786%	6.716	—	—	99.0

[a] Average of 4 determinations; SD = 0.022, CV = 2.1%.
[b] Average of 4 determinations; SD = 0.0026, CV = 2.5%.

Table 2. Collaborative results for AAS determination of mercury in mercury-containing drugs (mg/mL[a] or mg/g[b])

Sample[c]	Content, mg/mL[a] or mg/g[b]	Coll. 1	2	3	4	5	6	7
A	0.01	0.01	0.0097	0.0097	0.0094	0.0089	0.01	0.01
		0.01	0.0098	0.0097	0.0093	0.0094	0.01	0.01
B	0.10	0.10	0.106	0.104	0.106	0.103	0.10	0.099
		0.09	0.105	0.100	0.099	0.103	0.10	0.098
C	1.00	1.00	1.07	1.02	1.01	1.06	0.98	1.01
		0.99	1.06	1.02	1.03	1.06	0.98	1.01
D	50.00	48.9	49.5	50.0	51.0	56.9	49.9	51.4
		49.1	49.7	51.0	51.3	52.2	49.7	51.1
E	100.00	109.0	95.7	145.9	103.7	101.0	99.8	97.3
		101.0	96.9	145.8	104.7	98.0	98.1	97.3

[a] Samples A, B, and C.
[b] Samples D and E.
[c] Synthetic samples: A, thimerosal eye drops; B, phenylmercuric borate solution; C, thimerosal tincture. Commercial samples: D, ammoniated mercury ointment; E, mersalyl injection.

Table 3. Collaborative results for mercury content (%) of synthetic and commercial samples

Sample[a]	Collaborator 1	2	3	4	5	6	7
A	100	97	97	94	89	100	100
	100	98	97	93	94	100	100
B	100	106	104	106	103	100	99
	90	105	100	99	103	100	98
C	101	107	102	101	106	98	101
	99	106	102	103	106	98	100
D	97.8	99.0	100	102	119.8[b]	98.8	102.8
	98.2	99.4	102	102.6	104.4[b]	99.4	102.2
E	109	95.7	145.9[b]	103.7	101	99.8	97.3
	101	96.9	143.8[b]	104.7	98	98.1	97.3

[a] For identification of samples, see Table 2.
[b] Outlier by Dixon's test (4); excluded from statistical evaluation.

Results and Discussion

Most AAS methods for determining metals require that the analyte be present in solution. For samples containing organically bound metals, this is usually accomplished by digesting the sample with an oxidizing acid to destroy the organic component and release the metal in its ionic form. In the case of mercury-containing compounds, total destruction of the organic matter is impractical because a prolonged digestion can result in significant losses of the volatile mercury. When the AAS determination involves the flameless technique, hydrochloric acid can be used to cleave all types of organically bound mercury compounds except those with mercury-sulfur bonds (2). In the method pre-

Table 4. Statistical evaluation of results for mercury content (%) from 7 collaborators

Parameter evaluated (n = 66)[a]	Found
Mean mercury content, %	100.13
Repeatability SD	2.18
Repeatability CV, %	2.18
Reproducibility SD	3.38
Reproducibility CV, %	3.38

[a] Of a total of 70 determinations, 4 were outliers and were excluded from the evaluation (see Table 3).

sented here, a dilute mixture of hydrochloric acid–nitric acid was used because it cleaves a greater variety of mercury bonds and, in addition, it can be used with either the flame or the flameless mode of AAS.

To determine the applicability, precision, and accuracy of the proposed AAS method, various commercial and synthetic spiked and unspiked samples were analyzed in-house; the results are presented in Table 1. This table also shows that an unspiked commercial ophthalmic solution declared to contain 0.001% thimerosal as preservative contained no thimerosal when analyzed. In contrast, the same sample gave a satisfactory recovery after spiking. The apparent loss of thimerosal during storage has been documented previously (3).

The analytical results submitted by each collaborating laboratory are summarized in Table 2. The values for percent mercury from each of the 5 samples collaborated and the corresponding statistical evaluation are reported in Tables 3 and 4, respectively. Results from Collaborator 3, Sample 3 (mersalyl injection), and from Collaborator 5, Sample 2 (ammoniated mercury ointment), were outliers by Dixon's test (4) and were excluded from the statistical evaluation.

One collaborator experienced difficulties in obtaining a steady AAS signal when using the air–acetylene flame. This problem was probably due to the reduction of Hg(II) to Hg(I) or to elemental mercury from other components in the sample, which, if present, would have led to increased sensitivity and thus to erroneously high results (5). We have found that the addition of potassium dichromate to the test solution in amounts needed to produce a final potassium dichromate concentration of 0.2% yields the stable signal shown in Figure 1. To prevent this difficulty, all solutions analyzed by flame AAS should contain 0.2% potassium dichromate. As an added precaution, the mixing chamber of the spectrophotometer should be thoroughly cleaned before the analysis to remove Sn(II) and

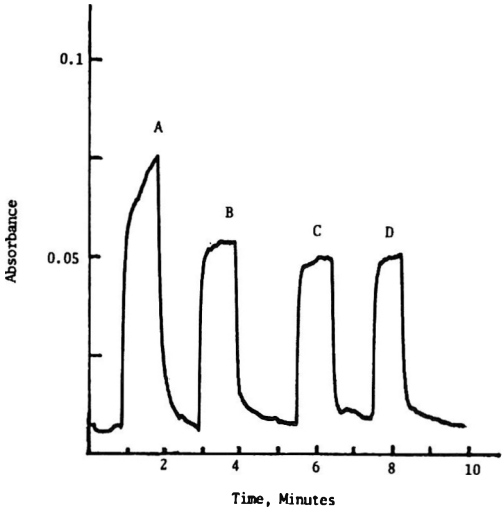

Figure 1. Effect of $K_2Cr_2O_7$ on AAS mercury signal in air-acetylene flame. Sample of ammoniated mercury ointment, 5%: A, no $K_2Cr_2O_7$ added; B, 0.2% $K_2Cr_2O_7$ added. Standard solution of $HgCl_2$: C, no $K_2Cr_2O_7$ added; D, 0.2% $K_2Cr_2O_7$ added.

other reducing agents, which also might cause an erratic signal if present in the chamber.

Recommendation

It is recommended that the proposed AAS method for determination of mercury in mercury-containing drugs be adopted official first action.

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PESTICIDE FORMULATIONS

High Performance Liquid Chromatographic Determination of Diclofop-Methyl

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Diclofop-methyl is diluted in acetonitrile-2% acetic acid (70 + 30) and chromatographed on a reverse phase C-18 column. The analyte is eluted by an acetonitrile-2% acetic acid mobile phase and detected at 280 nm. The method was validated by testing precision, linearity, and recovery. Precision and linearity were excellent (% RSD = 0.55, r^2 = 0.9997). A multiple standard addition to a pool of formulation samples showed the method to have excellent recovery (99%) and to be free of matrix interferences.

Diclofop-methyl (methyl 2-(4-(2',4'-dichlorophenoxy)-phenoxy) propanoate) is an EPA restricted use pesticide used for the post-emergence control of annual grassy weeds in soybeans, wheat, and barley. This herbicide is marketed as a 3 lb/gal. (35.49%) emulsifiable concentrate by the basic producer under the trade name Hoelon.

There are no published methods of direct analysis of this compound. Indirect methods used include bioassay (1) and thin layer chromatography of samples supplemented with ^{14}C-labeled standard (2–4). The basic producer of this pesticide uses an unpublished gas chromatographic assay in quality control.

METHOD

Reagents

(a) *Extractant.*—Acetonitrile–2% acetic acid (70 + 30).

(b) *Mobile phase.*—Acetonitrile–2% acetic acid (70 + 30), or as adjusted to give $k' \geq 2$ for analyte.

(c) *Reference standard.*—Hoelon, 99% (American Hoechst Corp., Rt 202–206 N, Somerville, NJ 08876).

Apparatus

(a) *High pressure liquid chromatograph.*—Varian Model 5060 equipped with Varian Model 8055 Autosampler (Varian Associates, Palo Alto, CA 94303), or equivalent. Perkin-Elmer LC 55B variable wavelength detector (Perkin-Elmer Corp., Norwalk, CT 06856), or equivalent.

(b) *Chromatographic column.*—Alltech C-18 10 μm column (Alltech Associates, 2051 Waukegan Rd, Deerfield, IL 60015), or equivalent. Chromatographic conditions: 10 μL loop; flow rate 1.5 mL/min; detection at 280 nm at 0.5 AUFS.

Procedure

Weigh standards and samples to contain 100 mg diclofop-methyl, dilute to 100 mL with extractant and chromatograph. Each 2 sample injections should be bracketed by standard injections which are averaged for calculation of sample concentration.

Calculations

% Active ingredient = $(P/P') \times (W'/W) \times (V/V') \times$ % purity of standard where P and P' = peak height of sample and standard, respectively; W and W' = weight of sample and standard, respectively; V and V' = final volume of sample and standard, respectively.

Method Evaluation

Precision of the method was tested by assaying 7 formulations in triplicate. Linearity was tested by chromatographing the standard at various dilutions. The method was tested for recovery and matrix effects by performing a standard addition in triplicate, at 2 levels, to a pool of formulation samples. Recovery was determined by calculating the amount of analyte found due to standard as a percentage of that added. The matrix effects were tested by plotting percent found vs amount added. The x-intercept corresponds to the amount that would have to be subtracted from the matrix to yield a 0% determination. Absence of a matrix effect is indicated when this intercept corresponds to the amount of analyte in the sample.

Results and Discussion

Figure 1 represents a typical chromatogram of Hoelon 3EC (35.49% diclofop-methyl). Retention time of the analyte peak was 9 min.

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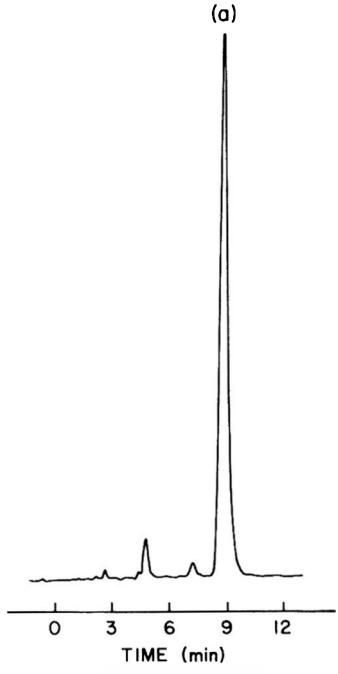

Figure 1. Chromatogram of diluted 35.49% diclofop-methyl (a) formulation. (retention time = 9 min).

Triplicate analysis of seven Hoelon 3EC formulations gave an average relative standard deviation (RSD = standard deviation/mean × 100) of 0.55% with the largest RDS being 0.8% (see Table 1). This level of precision indicates that the use of an internal standard is not necessary in this method. Linearity of peak height across 5 standard dilutions was excellent. Least squares analysis gave a coefficient of determination (r^2) of 0.9997.

Table 1. Results of triplicate analysis of 7 Hoelon 3EC formulations

Laboratory No.	% Found ± SD (% RSD)[a]
82-14	38.0 ± 0.3 (0.8)
82-15	38.0 ± 0.06 (0.2)
82-16	37.9 ± 0.06 (0.2)
82-447	37.9 ± 0.3 (0.8)
82-448	37.6 ± 0.2 (0.5)
82-601	36.3 ± 0.2 (0.6)
82-602	37.2 ± 0.3 (0.8)

[a] ± standard deviation (% relative standard deviation).

Mean recovery of standard from the formulation pool was 99% with a standard deviation of 6%. The standard deviation of percent recovery is inflated in this procedure by the variation in analysis of analyte in the formulation pool. The plot of percent found vs amount of standard added gave an x-intercept of −19.3 mg in the formulation pool. This corresponds well to the expected 19.45 mg, thus indicating a lack of matrix effect on our assay.

The results presented here show this assay to give good precision, linearity, recovery, and to be free of interferences from the tested sample matrix. These results support the use of this method to assay diclofop-methyl at least in the Hoelon 3EC formulation.

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Gas-Liquid Chromatography and Nitrogen-Phosphorus Detection of *N*-Nitroso-di-*n*-Propylamine in Trifluralin Products

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A method is described for the determination of trace levels of *N*-nitroso-di-*n*-propylamine (NDPA) in trifluralin herbicide formulations. Following sample extraction, NDPA is separated on a silica gel column and determined by gas-liquid chromatography with a nitrogen-phosphorus detector (NPD). A comparison between the results obtained using this detection system and the thermal energy analyzer (TEA) for 0.5-5 ppm levels of NDPA in 17 samples showed no significant differences. Close agreement was also demonstrated with the results obtained by high resolution gas-liquid chromatography/mass spectrometry (GLC/MS). The minimum detectable amount of NDPA with the NPD was 5 pg, for the TEA system 50 pg, and for high resolution GLC/MS 100 pg. A number of other nitrosamines were detected by all systems in one sample but these were not fully characterized. The nitrogen-phosphorus detector is useful for monitoring traces of NDPA in trifluralin when preceded by cleanup on a silica gel column.

N-Nitroso compounds have been demonstrated to be carcinogenic in several species of animals, and are therefore likely to be causally related to human cancer (1). The presence of *N,N*-dialkyl-nitrosamines in some widely used herbicidal formulations was reported by Ross et al. (2), and the general subject of nitrosamines in relation to pesticides has been reviewed more recently by Kearney (3). In the herbicide α,α,α-trifluoro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine (trifluralin), the nitrosamine corresponding to the dialkylamino group on the aromatic ring is *N*-nitroso-di-*n*-propylamine (NDPA); the presence of up to 154 ppm was reported originally by Ross et al. (2). Cohen et al. (4) found between 21 and 188 ppm NDPA in trifluralin before the production process was modified to reduce NDPA content to between 11 and 22 ppm. Subsequently, the level in technical trifluralin has been further reduced, in most cases to below 1 ppm. Since the regulatory limit in Canada is set at 1 ppm in technical trifluralin, this study was initiated to evaluate the methodology for this level of NDPA in formulated products.

Day et al. (5) reported a method for the analysis of nitrosamines in dinitroaniline herbicides, using a gas-liquid chromatographic (GLC)-thermal energy analyzer (TEA) system that gave a sensitivity of 0.1 ppm. The TEA system has the ability to specifically detect compounds that produce the nitrosyl radical following pyrolysis, and thus it is very specific for the determination of volatile nitrosamines, although some interferences have been reported (5, 6). In a comparison of some chromatographic systems which examined GLC/TEA, GLC-high resolution mass spectrometry, GLC-Coulson electrolytic conductivity detection, and thin layer chromatography, the TEA detector was reported to be more sensitive by a factor of about 50 (7). Webb et al. (8) reported that gas-liquid chromatography coupled with high resolution mass spectrometry is the method of choice for unequivocal identification of nitrosamines, with a sensitivity superior to all other techniques. Recently, the Hall electrolytic conductivity detector has been modified for the specific detection of nitrosamines in the presence of other nitrogen compounds (9), with <10 pg reported as the minimum detectable amount.

Most pesticide quality control laboratories have a gas chromatograph equipped with a hydrogen flame ionization detector which can be easily modified to a nitrogen-phosphorus selective detector (NPD). The present study compares results obtained using this system, preceded by silica gel column cleanup, with those obtained using the TEA detector.

METHOD

Apparatus

(a) *Gas-liquid chromatograph with NP detector.*—Hewlett-Packard 5710A fitted with nitrogen-phosphorus flame ionization detector and 1.85 m × 2 mm id glass column packed with 10% Carbowax 20M-1% NaOH on 80-100 mesh Chromosorb P. Operating conditions: inlet 200°C, column oven 160°C, and detector 300°C. Carrier gas flow: high purity helium, 30 mL/min. Detector gases: hydrogen, 3.0 mL/min; air, 50 mL/min.

(b) *Gas-liquid chromatograph/mass spectrometer.*—Perkin-Elmer Sigma 3 gas chromatograph with single glass jet separator and Kratos MS-50 mass spectrometer. GLC column: 1.85 m × 2

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mm id glass packed with 20% Carbowax 20M–1% NaOH on 80–100 mesh Chromosorb P. Temperatures: column 180°C; injector 200°C. Helium carrier gas flow 30 mL/min. Mass spectrometer conditions: resolution 10 000; ion current 500 μA; accelerator voltage 8000; source 200°C. Operation mode: single ion monitoring m/z 130.1106.

(c) *Gas-liquid chromatograph/TEA.*—Thermal energy analyzer Model 502A interfaced with Hewlett-Packard 5710A gas chromatograph equipped with 1.85 m × 2 mm id stainless steel column packed with 10% Carbowax 20M–1% NaOH on 80–100 mesh Chromosorb P. Operating conditions: inlet 200°C, column oven 160°C, interface 200°C; carrier gas, high purity helium, 30 mL/min. Operate thermal energy analyzer at pyrolizer temperature of 485°C using CTR gas stream filter (Thermo Electron Corp., Waltham, MA).

(d) *Chromatographic columns.*—30 cm long × 1.9 cm id, fitted with Teflon stopcocks.

(e) *Kuderna-Danish evaporators.*—Kontes Scientific Glassware.

(f) *Sealed vials.*—GLC Autosampler vials (Hewlett-Packard) or Mininert vials, 3.5 mL (Pierce Chemical Co.).

Materials

(a) *NDPA standard.*—Eastman Organic Chemicals or Sigma Chemical Co. Stock standard: 1 mg/mL. Measure 10 μL at 20°C (S.G. 0.9163, CRC Handbook) into ca 1 mL absolute ethanol in 10 mL low-actinic volumetric flask and dilute to volume with absolute ethanol. Working standards: 1 and 0.1 μg/mL. Dilute 100 and 10 μL respectively of the stock standard to 100 mL ethanol. Daily, transfer 1 μg/mL and 0.1 μg/mL working standards to a sealed vial. (Ready-diluted analytical standards are also available from Thermo Electron Corp.) Handle standards and samples in fume hoods with all appropriate safety precautions. Handle under red fluorescent lighting to avoid photodegradation of nitrosamines.

(b) *Solvents.*—All glass-distilled; ethyl ether contains 2% ethanol preservative.

(c) *Silica gel.*—E. Merck SG 60. Condition 3 days at 100°C.

Sample Cleanup

To prepare columns, slurry 25 g silica gel with ca 100 mL 1% ethyl ether in dichloromethane in 250 mL separatory funnel and drain into chromatographic column containing plug of glass wool and layer of sand. Top silica gel bed with layer of anhydrous sodium sulfate and drain solvent to top of sodium sulfate.

Technical materials.—Weigh ca 1 g sample into 50 mL beaker, dissolve and transfer quantitatively to cleanup column with small portions of 1% ethyl ether–dichloromethane (about 10 mL total), then elute with balance from total of 150 mL solvent mixture. Discard this fraction (containing trifluralin and other compounds) and proceed to elution of nitrosamines.

Liquid formulations (40%).—Accurately weigh ca 2.5 g sample by difference and transfer to cleanup column. Elute with 150 mL 1% ethyl ether–dichloromethane, and discard eluate. Proceed to elution of nitrosamines.

Granular formulations (5%).—Weigh 30 g sample into 100 mL amber glass bottle with Teflon-lined screw cap and shake with 75 mL 1% ethyl ether–dichloromethane for 15 min. Let separate and transfer 50 mL aliquot of extract onto cleanup column. Elute with an additional 100 mL solvent mixture and discard eluate. Proceed to elution of nitrosamines.

Elution of Nitrosamines

For all sample types, elute nitrosamines with 175 mL 4% ethyl ether in dichloromethane, collecting eluate in 250 mL Kuderna-Danish evaporator fitted with 10 mL 19/22 graduated receiving tube and size 121 24/40 Snyder column.

Sample Concentration

Add one anti-bumping granule and immerse evaporator in 48–52°C water bath to depth that gives evaporation rate of ca 100 mL/h. Seal top half of lower 19/22 joint with light film of silicone grease. After concentration of solvent to ca 5 mL, raise evaporator and cool, then rinse Snyder column and flask with ca 0.5 mL absolute ethanol in ca 3 mL dichloromethane. Remove column and flask from receiving tube, first making sure that outside of tube is thoroughly dry. Add another anti-bumping granule and fit receiving tube with 3 chamber Snyder column. Concentrate extract to ca 0.5 mL, and dilute to 1.0 mL with ethanol added dropwise through micro-Snyder column. Transfer aliquot to sealed vial for GLC analysis.

Gas-Liquid Chromatographic Analysis

Inject macro quantity (ca 1 μg) of NDPA to condition column. Subsequent responses for 1 μg standard injections should vary less than 5%. Inject 5 μL aliquot of sample extract at 1 × 4 or

other attenuation providing maximum sensitivity compatible with acceptable baseline noise. (Typically, 5 μL aliquots of 1 μg/mL and 0.1 μg/mL working standards will produce one-half full-scale deflection at attenuations 1 × 32 and 1 × 4, respectively).

Calculations

Nitrosamine (ppm) in trifluralin sample =

$$(R_{sam}/R_{std}) \times (W_{std}/W_{sam}) \times \text{purity of std}$$

where R_{sam} = detector response of sample injected; R_{std} = detector response of standard injected; W_{std} = weight (μg) nitrosamine standard injected; W_{sam} = weight (g) trifluralin injected. Weight of trifluralin is calculated from trifluralin (% w/w) contained in sample as determined by GLC/FID analysis.

Results and Discussion

The activated silica gel cleanup column was used in preference to the deactivated alumina used by Day et al. (5) because of better availability of the material and greater ease of preparation. The ethyl ether–dichloromethane solvent systems were chosen because of their lower boiling range, which helped reduce losses of nitrosamines in the evaporation step. The order of elution of 8 nitrosamine standards from a 25 g silica gel column, using 175 mL 4% ethyl ether–dichloromethane, was established by analysis of fractions of the eluate collected. *N*-Nitrosoethylmethylallylamin eluted first, followed by *N*-nitrosodibutylamine, -butylpropylamine, -butylethylamine, -di-*n*-propylamine, -diethylamine, and last, -dimethylamine. The last compound was only partially eluted; use of 10% ethyl ether–dichloromethane is required for quantitative recovery. This order of elution is reported as useful analytical information, even though clear separations were not obtained between all standards.

Crosby (10) has stated that solvent evaporation is the single most critical step in nitrosamine analysis, and great care must be exercised to avoid unacceptable losses. Most workers favor using the Kuderna-Danish evaporator fitted with a micro-Snyder column, in preference to the rotary evaporator, and use a small volume of a higher boiling solvent as keeper, which in this study was ethanol. Concentration by using a rotary evaporator with a water aspirator pump and a water bath at 40°C was used for screening large numbers of samples. Losses of NDPA were minimal, but recoveries of *N*-nitroso-di-*n*-ethylamine were about 80% and of *N*-nitroso-di-*n*-methylamine about 50% in analyses for nitrosamines in pesticides other than trifluralin. Recoveries depend largely on the performance and condition of the rotary evaporation system, particularly the vacuum seals.

Table 1. Recovery of standard NDPA added to sample of trifluralin 40% emulsifiable concentrate of known NDPA content

ppm NDPA			
Base level	Added	Total	% Recovery
1.2	0.6	1.8	96, 115, 100
1.2	1.2	2.4	96, 100, 91
1.2	2.4	3.6	101, 110, 104
1.2	3.6	4.8	101, 113, 110

Initially, a Chromosorb 103 porous polymer column was used for gas chromatography because this material is quite selective to the lower molecular weight nitrosamines. However, this column showed a low efficiency, and its power to resolve closely related nitrosamines declined with repeated use. Moreover, when connected with a mass spectrometer for confirmatory purposes, it produced an unacceptable degree of bleed. Chromosorb P treated with 1% NaOH and coated with 10% Carbowax 20M was a more efficient and stable packing material.

To study the recovery of NDPA at levels above 1 ppm, 8 replicates of a 40% EC liquid formulation were analyzed, using the NP detector, and showed a mean NDPA level of 1.2 ppm, with a relative standard deviation of ±5%. This material was then fortified with several levels of standard NDPA, and analysis gave an average recovery of 103.2% (Table 1).

To fortify samples at the 0.1 ppm level, it was necessary to produce nitrosamine-free trifluralin, because all samples were contaminated above this level. This was prepared by passing a solution of technical trifluralin through a scaled-up silicic acid cleanup column, then adding to the nitrosamine-free material the appropriate solvent and adjuvants to give a 40% EC formulation. Analysis of this fortified material gave recoveries of 90, 108, and 95% at the 0.2 ppm level, and 106, 106, and 106% at the 0.1 ppm level.

Recovery studies were also carried out on trifluralin 5% granular formulation. Recoveries were 103, 75, and 59% at the 0.32 ppm added NDPA level; 94% at 0.64 ppm; 97 and 76% at 0.96 ppm; and 97 and 97% at the 1.29 ppm level.

Samples already contaminated at the 0.62 ppm level were fortified with additional NDPA and

Table 2. Comparison of results for NDPA (ppm) in trifluralin, using NP and TEA detectors

NP detector	TEA detector
0.76, 0.77	0.80, 0.75
0.74, 0.76	0.75, 0.77
0.60, 0.59	0.64, 0.65
0.80, 0.92	0.98, 0.97
0.60, 0.65	0.69, 0.69
4.15, 4.82	3.92, 4.05
1.01, 0.84, 1.02	0.96, 1.00
2.09, 2.15	2.20, 2.27
0.81, 0.87, 0.83	0.91, 1.0
2.38, 2.50	2.89, 3.05
2.34, 2.08	1.96, 2.10
2.15, 2.57, 2.29, 2.21	2.19, 2.14
1.17, 1.22	1.08, 1.03
1.10, 1.11	0.99, 1.05
1.42, 1.49	1.44, 1.39
5.04, 5.35	4.15, 4.08
1.19, 1.22	1.16, 1.12

a least squares plot of results indicated an average recovery of 92%.

To compare the responses of the NP and TEA detectors, samples of trifluralin emulsifiable concentrate were extracted and duplicate injections were made on each detection system. A comparison of responses is shown in Table 2. Statistical analysis, using the paired *t*-test, showed no differences between the means of the 2 sets of results.

Gas-liquid chromatography-high resolution mass spectrometry was used for confirmation of the levels and identity of nitrosamines. Table 3 shows comparison of results obtained by GLC/MS with the other 2 detection systems. Again, no significant differences are demonstrated.

Based on the criterion that the minimum detectable amount (MDA) of a compound gives a recorder response twice the baseline noise, the MDA for the TEA detector was 50 pg and for the NP detector, 5 pg. For high resolution GLC/MS, the minimum detectable amount of NDPA at a resolution of 10 000 was 100 pg. Response with the NP detector was linear from 0.1 to 10 ng.

Figure 1 shows a typical chromatogram of a trifluralin sample extract, recorded from the NP detector. Figure 2A shows the chromatogram of a typical extract which exhibits 5 significant peaks with the NP detector. Similar traces were obtained by GLC/MS where m/z 29 9979 (NO.) was monitored (Figure 2B) and when the TEA response for the nitrosyl radical was recorded (Figure 2C), thereby providing the strongest evidence that all 5 peaks are nitrosamines.

Figure 3 shows the chromatogram produced

Table 3. Determination of NDPA (ppm) in trifluralin by GLC/MS compared with results using NP and TEA detectors

Sample	GLC/MS	NPD	TEA
1	3.2	3.3	2.9
2	2.2	1.7	1.8
3	1.1	1.7	1.4
4	2.0	1.5	1.5
5	2.0	1.5	2.2
6	1.7	1.6	1.4
7	1.2	1.7	1.3

with single ion monitoring at m/z 130.1106 which shows 2 responses for the molecular ion corresponding to a C_6-nitrosamine. The same sample produced 2 apparent nitrosamine peaks having similar retention times with the NP detector.

The presence of other nitrosamines in trifluralin was reported by Day et al. (5) who identified a peak eluting after NDPA as a C_7-nitrosamine by GLC/MS. These workers also reported a peak at a retention time of 1.8 relative to NDPA under their conditions, which was identified as

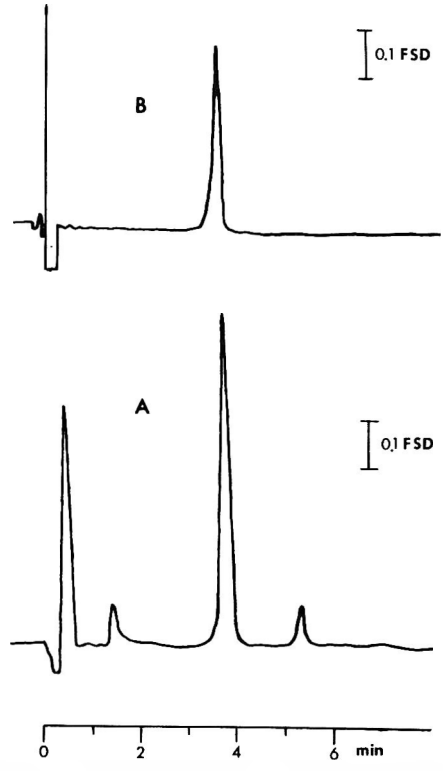

Figure 1. A, chromatogram of typical trifluralin extract, using NP detection; B, chromatogram of NDPA standard.

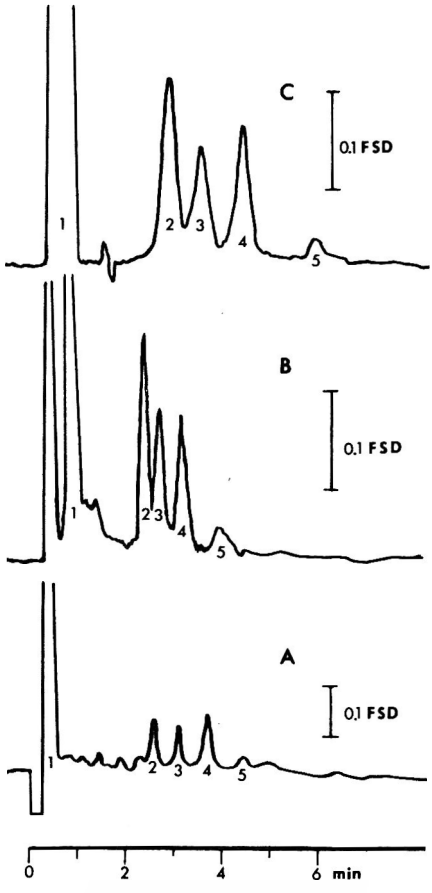

Figure 2. Chromatograms of trifluralin extract with 5 significant peaks: A, response using NP detector; B, response with TEA detector; C, GC/MS response when monitoring the NO ion (m/z 29 9979).

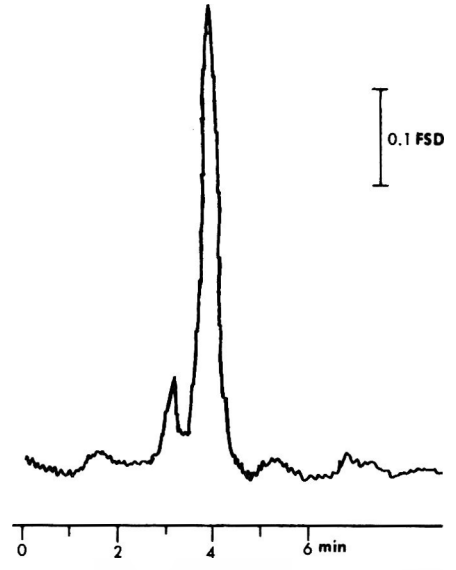

Figure 3. GLC/MS response of trifluralin extract when monitoring the single ion m/z 130.1106, showing 2 peaks corresponding to C_6 nitrosamines.

a nitramine compound. The identity as well as quantitation of *N*-nitrosamines other than NDPA is of concern where limits are set in terms of total *N*-nitrosamines in a product. In the present study, no further investigation into the identity of *N*-nitrosamines other than NDPA was made because they appear at significant levels only occasionally, and an estimate of the total *N*-nitrosamine content did not exceed 1 ppm.

This study has demonstrated that the NP detector, when used following silica gel cleanup, can be used with a considerable degree of confidence as an inexpensive alternative to a TEA or GLC/MS system for monitoring levels of NDPA in trifluralin products. NP detection offers a greater degree of sensitivity than most other available detectors. After analysis of several hundred trifluralin samples from a number of manufacturing sources, we have found no interferences which gave false positive results for NDPA. Nevertheless, confirmation by GLC/TEA or GLC/MS will be desirable when results above the regulatory levels are discovered.

Acknowledgments

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Liquid Chromatographic Determination of Glyphosate Technical and Its Formulation: Collaborative Study

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An HPLC method for the determination of glyphosate in formulation and technical samples has been subjected to a collaborative study with 12 laboratories participating. This method requires no sample pretreatment. Samples were dissolved in mobile phase, injected directly using a fixed-volume loop, and quantitated by an external standard technique. Compounds were separated on a strong anion exchange column with a water-methanol (96 + 4) mobile phase that was 0.0062M in KH_2PO_4 and adjusted to pH 1.9 with 85% H_3PO_4, and detected with a variable wavelength UV detector at 195 nm. Calculations were made using peak areas. The collaborative study involved 3 pairs of matched samples with single determinations on each sample: Roundup herbicide, a technical intermediate, and technical glyphosate. The coefficients of variation for the 3 pairs were 1.70, 0.88, and 0.90%, respectively. The method has been adopted official first action.

Glyphosate, *N*-(phosphonomethyl)glycine, is the active ingredient in Roundup® herbicide. This formulation is a water-soluble, nonselective herbicide that is applied to foliage. Acceptance of this herbicide worldwide has necessitated development of an accurate, precise analytical method for determining glyphosate. Glyphosate is nonvolatile and, because the formulation is a water-based system, derivatization and analysis by gas chromatography was not a desirable approach for method development. A method based on forming a colorimetric derivative followed by spectral analysis is being used by some laboratories (1). This method has been used by industry and regulatory agencies with some degree of success, but the method is quite lengthy, and accuracy and reproducibility are not satisfactory. For these reasons, we decided that high pressure liquid chromatography (HPLC) would be a viable approach to developing a method. Earlier work on this approach was reported by Burns and Tompkins (2).

The HPLC method described here has been used in the author's laboratory for over 5 years on commercial formulations and in process control. Within our laboratory, the method has a standard deviation of ±0.13% on formulations with 41% active ingredient. These precision data were generated on the same sample analyzed by 4 different technicians over 5 months. The method has no known interferences, is linear over the range studied, and is quite rugged. Over 1000 injections have been made on one column before any significant loss of resolution was observed. Analysis takes less than 1 h, compared with several hours for other less accurate methods, such as the spectral method referred to earlier (1).

Development of Method

The method has been used in the author's laboratory for over 5 years. During this time, it has been evaluated for precision, linearity, interferences, recovery, and sample stability. Analytical results indicate an improvement over other methods (1–3) and it is faster with improved precision. A typical chromatogram for a Roundup herbicide is shown in Figure 1. The chromatogram illustrates good peak symmetries and separation from known typical impurities. The early eluting peaks are due to the emulsifier, and a small impurity peak appears just after the glyphosate. A doublet peak appears in the 10–12 min range; this doublet is 2 isomers of another impurity. Operating parameters are adjusted for baseline separation of glyphosate and the small impurity that follows. The parameters given in the method should achieve this separation.

The precision of the method was demonstrated by analysis of the same sample monthly for 5 months by 4 different technicians. The data on 3 sample types are shown in Table 1. The coefficients of variation (CV) for these 3 samples were 0.32, 0.54 and 0.48%, respectively. The CV for each technician was essentially equivalent to the CV for all data when pooled for that sample.

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The recommendation of the Associate Referee was approved by the General Referee and by Committee A and was adopted by the Association. See the Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, 334; 411.

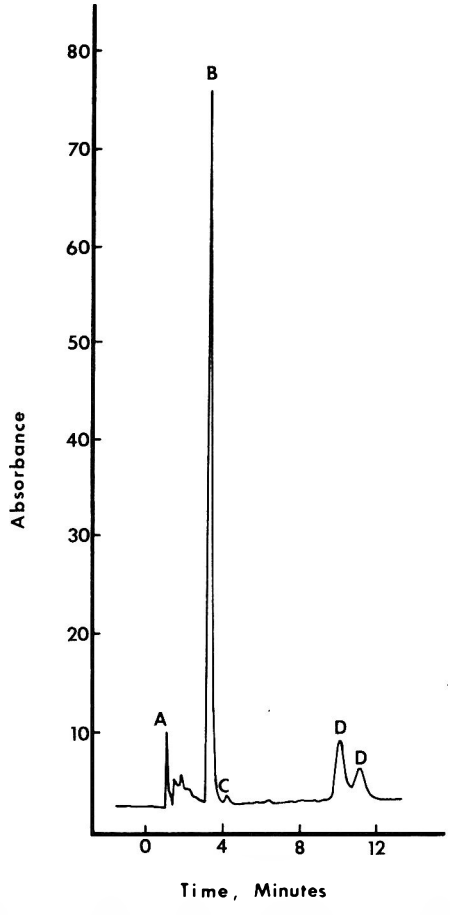

Figure 1. HPLC separation of surfactant (A), glyphosate (B), impurity 1 (C), and 2 isomers of impurity 2 (D) on glyphosate herbicide sample.

The linearity was tested by preparing standards up to 500 mg in 100 mL mobile phase. The area response vs concentration was linear over this range and passed through the origin. Above 500 mg, the curve starts to flatten. We have determined that consistent results are obtained when the sample concentration is similar to the standard and in the range suggested in the method.

Possible known interferences from raw materials, reaction by-products, etc., have been checked, and no interferences were found. This was further confirmed by previous work (2), which showed that retention times could be predicted by the pKa of the compound. The higher the pKa, the shorter the retention time. The known compounds most likely to interfere have pKa values different from that of glyphosate.

A recovery test was carried out by making 4 small laboratory batches of Roundup formulation with standard glyphosate and all normal raw materials used in the formulation. Samples of these batches were weighed so that the analytical samples for these 4 batches contained 350, 375, 400, and 425 mg glyphosate. Recovery was 347, 377, 396, and 423 mg, respectively (99.0–100.5%). This is acceptable recovery and also confirms the absence of interferences.

The typical chromatogram shown in Figure 1 is possible under conditions stated in the method. These conditions are recommended for routine use. Slight modifications in either mobile phase salt concentration or pH can change retention times and affect separation. Columns should be replaced when baseline separation cannot be achieved between glyphosate and the small impurity which follows. Modification of the mobile phase in an attempt to improve separation on a used column results in loss of efficiency and precision and is not recommended.

There are several other observations about the method that affects results. Formulation samples contain surfactant and, in water solution, bubbles form easily. Any bubbles present should be removed from the syringe before injection because they will affect reproducibility of the fixed volume loop and external standard quantitation. Some loss of efficiency due to initial settling of the column packing has been noted. Repacking the inlet end with support restores efficiency. Addition of 4% methanol to the mobile phase retards bacterial growth and improves shelf life. Without methanol, shelf life is reduced.

Collaborative Study

Three sets of matched sample pairs together with standard glyphosate, a practice sample, and detailed guidelines were sent to 12 collaborators. The matched pairs were similar but not duplicate samples: Samples A and B were Roundup formulation, C and D were technical intermediates, E and F were solid technical glyphosate. A typical chromatogram was supplied and collaborators were instructed to vary conditions, if necessary, to achieve separation for the practice sample. Collaborators were requested to report any changes necessary to achieve separation. Collaborators were instructed to dry the standard and solid technical samples and to make only single determinations after analysis of the practice sample. Data sheets were provided for raw data such as sample and standard weights and areas. Each was asked to submit a chromatogram

Table 1. Precision study on glyphosate samples (% isopropylamine salt of glyphosate)

Month	Analyst 1	2	3	4	Overall[a]
			Roundup		
1	41.15	41.09	41.00	41.33	
2	41.26	41.44	41.38	41.17	
3	41.47	41.08	41.25	41.30	
4	41.21	41.38	41.35	41.35	
5	41.17	41.38	41.21	41.35	
Av.	41.25	41.27	41.24	41.30	41.25
SD	0.13	0.17	0.15	0.08	0.13
CV,%	0.32	0.41	0.36	0.19	0.32
			Technical Intermediate		
1	62.52	62.46	62.79	62.85	
2	62.60	62.24	62.95	62.75	
3	62.36	63.11	62.73	63.07	
4	62.62	63.07	63.36	63.71	
5	62.78	62.90	63.01	62.64	
Av.	62.58	62.76	62.97	63.00	62.83
SD	0.15	0.39	0.25	0.43	0.34
CV,%	0.24	0.62	0.40	0.68	0.54
			Technical Glyphosate		
1	96.30	95.60	95.83	96.74	
2	96.36	95.40	96.58	95.53	
3	96.38	96.30	96.05	96.90	
4	96.83	96.30	96.76	96.43	
5	96.29	96.40	96.80	96.26	
Av.	96.43	96.00	96.40	96.37	96.30[b]
SD	0.23	0.46	0.44	0.53	0.46
CV,%	0.24	0.48	0.46	0.55	0.48

[a] Pooled data using all 20 results.
[b] % glyphosate.

for Sample A. In general, instructions were followed closely. Any deviations from the method are noted below.

Glyphosate (*N*-Phosphonomethyl Glycine) Technical and Formulations

Liquid Chromatographic Method

Official First Action

6.D22 *Principle*

Samples are dissolved in phosphate buffer mobile phase and injected directly into ion exchange chromatogc system using fixed vol. loop. Peak area response as measured by UV detector is quantitated by external std technic.

6.D23 *Apparatus and Reagents*

(a) *Liquid chromatograph.*—Able to generate over 1000 psi and measure *A* at 195 nm.

(b) *Loop injector.*—Rheodyne Model 7120 syringe loading (Supelco, Inc.), or equiv.

(c) *Strip chart recorder.*—Houston Instrument 10 mV full scale (Industrial Scientific, PO Box 60002, Houston, TX 77060), or equiv.

(d) *Electronic integrator.*—Capable of handling detector output.

(e) *Chromatographic column.*—25 cm × 4.6 mm id, 1/4 in. od, strong anion exchange, e.g., Partisil 10 SAX (available from Whatman, Inc., 9 Bridewell Pl, Clifton, NJ 07014).

(f) *Methanol.*—HPLC grade (available from Burdick & Jackson Laboratories, Inc.).

(g) *Water.*—HPLC grade (available from Burdick & Jackson Laboratories, Inc.). Use thruout.

(h) *Potassium dihydrogen phosphate.*—Primary std grade (available from Fisher Scientific Co.).

(i) *Phosphoric acid.*—85%, reagent grade (available from Fisher Scientific Co.).

(j) *Glyphosate std.*—Monsanto Co.

(k) *Mobile phase.*—Dissolve 0.8437 g KH_2PO_4

in 960 mL H_2O. Add 40 mL MeOH and mix well. Using pH meter buffered at pH 2.0, adjust pH to 1.9 with 85% H_3PO_4. Filter and degas before use.

6.D24 ***Preparation of Standard***

Accurately weigh ca 400 ± 10 mg glyphosate std (dried 2 h at 105°) into 100 mL vol. flask. Dil. to vol. with mobile phase and stir to dissolve (30 min may be required to dissolve std). Soln is stable ≥1 week.

6.D25 ***Preparation of Sample***

Accurately weigh sample contg ca 400 mg glyphosate into 100 mL vol. flask contg ca 50 mL mobile phase. Dil. to vol. with mobile phase and mix well.

6.D26 ***Determination***

Adjust operating parameters so that glyphosate elutes at 2.5–4.0 min. Maintain all parameters consistent thruout std and sample analysis. Typical values are as follows: flow rate 2.3 mL/min; pressure ca 1200 psi; chart speed 0.5 cm/min; *A* range 0.2 AUFS; column temp. ambient; injector vol. 50 μL.

Let mobile phase flow thru system until steady baseline is obtained; 1 h may be required for new column. When new columns are installed or instrument has not been used for 24 h, make at least 6 rapid injections of std soln; then inject std soln until peak areas for successive injections agree ±1%. Then inject sample soln until peak areas for successive injections agree ±1%. Let all components from samples elute (ca 10–12 min) before making next injection.

6.D27 ***Calculation***

Average peak areas from 2 successive injections that agreed ±1% from both std and sample solns.

$$\% \text{ Glyphosate} = (R/R') \times (W'/W) \times P$$

where R = av. peak area of sample; R' = av. peak area of std; W = mg sample; W' = mg std; and P = % purity of std. To convert % glyphosate to isopropylamine salt, multiply by 1.3496.

Results

The collaborative test of a closely matched pair and interpretation of results as outlined by Youden and Steiner (4) was followed. In addition, published work by Pearson (5) and private communications with E. Glocker, statistical consultant to AOAC, were also used for interpretation.

Table 2 shows the results from all laboratories. The ranking test (4) was applied and all laboratories fell within limits; thus no laboratory was discarded as an outlier due to systematic error. Several pairs of results were rejected as outliers according to confidence limit calculations that follow: Limits for individual measurements

Table 2. Collaborative results for HPLC analysis of glyphosate

	Wt % isopropylamine salt				Wt % glyphosate	
Coll.	A	B	C	D	E	F
1 (AR)	41.2	41.0	61.8	62.6	97.7	97.9
2	44.7	43.9[a]	62.1	63.2	103.8	100.9[b]
3	42.5	41.4	62.6	62.7	98.8	98.2
4	41.8	41.2	63.5	62.0	97.2	96.7
5	41.8	41.4	62.8	63.4	98.2	98.4
6	41.4	41.0	62.2	63.0	96.6	97.8
7	43.6	41.5	62.9	62.6	96.5	96.6
8	41.8	42.3	60.5	63.1[b]	96.8	97.2
9	41.9	42.1	69.1	66.0[b]	98.6	99.5
10	42.6	43.3	62.7	63.6	94.7	98.3[b]
11	38.3	41.6[b]	62.6	62.5	98.6	97.4
12	41.8	41.2	62.6	63.1	97.4	98.5
Pairs	10		10		10	
Mean	42.08	41.64	62.58	62.87	97.64	97.82
S_r	0.57		0.55		0.55	
S_b	0.42		—[c]		0.69	
SD = $\sqrt{S_r^2 + S_b^2}$	0.71		0.55		0.88	
CV, %	1.70		0.88		0.90	

[a] Pair omitted by Dixon test (6).
[b] Outlier pairs at $P = 0.02$ by Pearson (5).
[c] Negative S_b^2 by chance. See Ref. 4.

were obtained by mean $\pm t \times S_d$, where the mean is that of a given sample (either one of the pairs), t = a probability of 0.02, and $S_d = \sqrt{S_r^2 + S_b^2}$, which is the standard deviation of a single measurement. Limits for sums are also based on the mean $\pm t \times S_d$, where the mean is that of the sum of a matched pair, t = a probability of 0.02, and S_d (Youden and Steiner (4)) = $\sqrt{S_r^2 + 2S_b^2}$. Limits for differences between 2 measurements ($N = 2$) were obtained by mean difference $\pm$ [$1.128(S_r) \pm t(0.8525)$] for $N = 2$, where $1.128(S_r)$ is the relationship between the mean range and the standard deviation ($1.128(S_r) = \bar{R}$ when $N = 2$), and 0.8525 is the standard deviation of ranges (S_R when $N = 2$). The values 1.128 and 0.8525 are from Pearson (5). In Table 2, sample pair A and B was omitted for Collaborator 11 as outliers on individual results for A and for the difference on A and B; sample pair C and D for Collaborator 8 was an outlier because the individual result of C was out of limits; sample pair C and D for Collaborator 9 was omitted as a gross outlier; sample pair E and F was an outlier for Collaborators 2 and 10 because the sum for Collaborator 2 and the difference for Collaborator 10 were out of limits. The results for Collaborator 2 on sample pair A and B were also omitted as outliers by the Dixon test (6).

The remaining results in Table 2 were subjected to a statistical evaluation for precision as described by Youden and Steiner (4). The evaluation was based on a closely matched pair similar in nature and similar amounts of active ingredient present. Results show that the standard deviation (SD) when combining Samples A and B was 0.71 with a CV of 1.70%; when Samples C and D are combined, S_b is negative by chance and SD was 0.55 with a CV of 0.88%; the combination of Samples E and F had an SD of 0.88 and the CV was 0.90%. This gave an overall average CV equal to 1.16% for all 3 sample types.

The collaborators made various changes from the recommended procedure. Several collaborators reported that changes in the mobile phase were necessary to achieve the desired separation. These were only slight changes in either the salt concentration or pH. One collaborator added 6% methanol to the mobile phase rather than the specified 4% by volume. Another common change was a different size sample loop; 2 collaborators used a 10 and another a 20 μL loop. In the latter case with a 20 μL loop, the sample concentration was increased 2.5 times to compensate for the smaller injection volume. Another deviation from the method was use of a sample weight out of the recommended range. This was not a serious deviation because no weights used were outside the linear portion of the curve. Collaborators 3 and 8 used peak height rather than peak area for calculations. Their results show good agreement with the other collaborators and thus peak height could be an alternative if integration capabilities are not available. Seven collaborators reported difficulty in obtaining 1% reproducibility for areas from one injection to another. In our laboratory, we normally do better than 1%, usually around 0.5%. Thus, experience with the method should definitely improve reproducibility of injections. One collaborator made only single injections; however, no results from that collaborator were outliers, so results were included in the statistical evaluation.

The method collaboratively studied is rugged and has good precision. The precision remained consistent even with the changes such as peak height vs area, size of sample injection, and modifications of mobile phase, and thus is a demonstration of method ruggedness.

Recommendation

It is recommended that the HPLC method for determining glyphosate technical and its formulations be adopted official first action.

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Liquid Chromatography of Liquid Formulations Containing 2,4-Dichlorophenoxyacetic Acid, Dicamba, and 2-(2-Methyl-4-chlorophenoxy)propionic Acid as Their Salts

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A high pressure liquid chromatographic method has been developed for liquid herbicide combinations that contain different combinations of 3 active ingredients including 2,4-dichlorophenoxyacetic acid (2,4-D), 2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP), and dicamba. A reverse phase column in the ion suppression mode and a binary solvent system separate all 3 herbicides quantitatively on a single chromatogram. The internal standard solution may contain 2 internal standards, salicylic acid and butyrophenone, for use with certain combinations of the herbicides. The solvent system resolves the compounds of interest from all significant impurities.

A variety of herbicides for yard use are available to consumers in retail stores today; the most common active ingredient is 2,4-dichlorophenoxyacetic acid (2,4-D). Formulations of 2,4-D as a single active ingredient are seldom available. Rather, many formulations are combinations of 2 or 3 active ingredients, ordinarily blends of 2,4-D with 3,6-dichloro-*o*-anisic acid (dicamba) and/or 2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP). These combinations occur not only in the liquid spray but also in weed-and-feed products, and are increasing in numbers. Thus, the pesticide analytical chemist has a complex and demanding task when attempting to separately quantitate each active ingredient.

Reverse phase high pressure liquid chromatography (HPLC) in the ion suppression mode has been very useful in separating isomers and other impurities in the AOAC HPLC methods for chlorophenoxy herbicides (1, 2); however, the 10 μm/25 cm column specified does not have the required efficiency to separate active ingredients, internal standard, and prominent impurities associated with each technical product in the combination formulations. In searching for a column that would have the necessary efficiency, a small particle size, higher carbon-loaded ODS stationary phase may be unsatisfactory because of higher molecular weight species that elute in an unacceptable length of time with accompanying zone spreading. In addition, high pressure combined with the small particle size of 5 μm or less would result in problems such as plugging filters, leaks, and shorter column life.

A column with improved efficiency has been found which separates each active ingredient quantitatively on a single chromatogram. The reverse phase column selected has a carbon loading of approximately 10% and residual silanols "capped" with a shorter chain length organosilane. Desirable characteristics of this column for use with high ionic strength buffers are increased efficiency, compared with the column specified for the present AOAC chlorophenoxy methods; virtually complete coverage of silica gel surface silanols, and, therefore, longer life expectancy for the column; low back-pressure of approximately 2200 psi, which includes about 500 psi contributed by the guard column.

Development of Method

Impurities in the technical product must be considered in developing an analytical method for a commercial herbicide. In the case of a formulation that contains a single herbicide there is greater latitude in selection of solvents, columns, and instrumental parameters. As the formulation increases in complexity, the available options diminish in number. The ratio of percentages claimed is a criterion in determining the relative contribution of impurities in the formulation from each technical product when 2 or 3 herbicides are blended. For example, the percentage of 2,4-D claimed can be 10 times greater than the claim for dicamba. Therefore, it becomes important to separate the relatively minor impurity, *p*-chlorophenoxyacetic acid (*p*-CPAA) from 2,4-D, which will co-elute with dicamba when using only 33% acetonitrile mobile phase as an isocratic solvent. To accomplish this separation, an initial mobile phase containing 22% acetonitrile is necessary.

In this work, attention was directed at developing methodology for the most complicated

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Table 1. Elution of combination herbicides, impurities, 2 internal standards, and *p*-bromophenol as marker, using acetonitrile–water (33 + 67) pH 2.68–2.70 as eluant

Time, min	Elution order	Solute[a]	
	1	3,6-DCSA	3,6-dichlorosalicylic acid
4.9	2	internal std	salicylic acid
5.5	3	PPA	phenoxypropionic acid
	4	o-CP	o-chlorophenol
6.1	5	*o*-CPAA	o-chlorophenoxyacetic acid
6.5	6	*p*-CPAA	*p*-chlorophenoxyacetic acid
6.6	7	dicamba	3,6-dichloro-o-anisic acid
	8	*p*-CP	*p*-chlorophenol
	9	3,5-DCSA	3,5-dichlorosalicylic acid
7.3	10	2,6-D	2,6-dichlorophenoxyacetic acid
8.8	11	2-CPPA	2-chlorophenoxypropionic acid
9.0	12	2-MPPA	2-methylphenoxypropionic acid
9.1	13	4-CPPA	4-chlorophenoxypropionic acid
9.2	14	*p*-Br-P	*p*-bromophenol (marker)
	15	2,6-DCP	2,6-dichlorophenol
10.3	16	2,4-D	2,4-dichlorophenoxyacetic acid
10.3	17	2,6-DCPPA	2,6-dichlorophenoxypropionic acid
10.7	18	2-M-6-CPPA	2-methyl-6-chlorophenoxypropionic acid
11.3	19	3,5-Dicamba	3,5-dichloro-o-anisic acid
12.8	20	—	unknown
13.4	21	2-M-4-CP	2-methyl-4-chlorophenol
13.6	22	2,4-DCP	2,4-dichlorophenol
15.8	23	2,4-DCPPA	2,4-dichlorophenoxypropionic acid
16.2	24	MCPP	2-(2-methyl-4-chlorophenoxy)propionic acid
17.7	25	2,6-DM-4-CPPA	2,6-dimethyl-4-chlorophenoxypropionic acid
18.9	26	2,4,6-TCPAA	2,4,6-trichlorophenoxyacetic acid
20.7	27	2-M-4,6-DCPPA	2-methyl-4,6-dichlorophenoxypropionic acid
	28	2,4,6-TCP	2,4,6-trichlorophenol
23.5	29	internal std	butyrophenone

[a] Compounds of interest and internal standards are underlined.

situation—the ternary formulation. Basic suppliers (The Dow Chemical Co., Midland, MI and Diamond Shamrock Corp., Tuscaloosa, AL) provided reference compounds which represent all known impurities in technical formulations. These were used to evaluate columns and mobile phases for their resolving power. Optimum conditions using a binary solvent system are acetonitrile–water (22 + 78) pH 2.68–2.70 as the initial solvent for eluting dicamba, and then acetonitrile–water (33 + 67) pH 2.68–2.70 for eluting the remaining solutes, including 2,4-D and/or MCPP, and the internal standard butyrophenone. Table 1 shows the elution order using the latter solvent (33% acetonitrile). Table 2 contains a list of typical formulations, currently in use, classified for the purpose of the assay method. If dicamba is not present, 33% acetonitrile can be used as an isocratic solvent.

We attempted to use the newer 5 μm columns to improve resolution and thereby circumvent the use of a binary mobile phase solvent system. Two columns, Whatman 5/25 ODS-3 and IBM Octadecyl (C_{18}) RP 5 μm/25 cm, were tried using different amounts of acetonitrile in the mobile phase. Using 37% acetonitrile–63% water mobile phase buffered to pH 2.68, it was possible to separate *p*-chlorophenoxyacetic acid from dicamba. Unfortunately, the 2,6-D impurity (from 2,4-D) moved under dicamba peak. In general, the 5 μm columns did not resolve peaks any better than did the 10 μm ODS-3, and there was the added disadvantage of having to use high pressure to attain a reasonable flow. Two other higher capacity columns were tried, Partisil 10 ODS-2 and 5 μm/25 cm Ultrasphere ODS. Results were unsatisfactory. The Partisil 10/25 ODS-3 column, specified in the method, offered the best compromise among resolution, speed, and capacity. No other column should be substituted without prior evaluation with respect to separation requirements because there may be co-elution problems (bias) with peaks of interest and impurities.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Fitted with 5000 psi pressure gauge and with 2.0 mL/min flow rate

Table 2. Typical combinations herbicide in formulations[a]

Formulation	Claim, %	Equiv. acid, %	Ratio	Mobile phase
Class I—Dicamba and 2,4-D:				
Dicamba	0.31	0.25	0.25	binary solvent:
2,4-D alkanolamine salt	1.66	1.00	1.00	acetonitrile–water (22 + 78) pH 2.70
Dicamba	3.42	2.84	0.20	acetonitrile–
2,4-D	17.12	14.21	1.00	water, (33 + 67) pH 2.70
Dicamba	3.18	2.64	0.25	
2,4-D	12.72	10.57	1.00	
Class II—Dicamba, 2,4-D, and MCPP:				
Dicamba	0.65	0.54	0.10	binary solvent:
2,4-D	6.27	5.2	1.00	same as Class I
MCPP	2.84	2.35	0.45	
Dicamba	1.20	1.00	0.19	
2,4-D	6.25	5.20	1.00	
MCPP	10.83	8.95	1.72	
Dicamba	1.28	1.07	0.40	
2,4-D	3.23	2.69	1.00	
MCPP	10.59	8.76	3.26	
Class III—2,4-D and MCPP:				
2,4-D	0.75	0.51	1.00	isocratic solvent:
MCPP	0.75	0.50	1.00	acetonitrile–water (33 + 67) pH 2.70
2,4-D	11.50	9.6	1.00	
MCPP	11.6	9.6	1.00	

[a] Active ingredients are dimethylamine salts except where noted.

capability; 280 nm UV detector; line filter in eluant reservoir; injection valve volume 10 μL; 10 mv full scale deflection strip chart recorder. Operating conditions: flow rate 2.0 mL/min, chart speed 0.5 cm/min, detector 280 nm, injection volume 10 μL, constant ambient temperature ≤76°F (troublesome bubbles form in acetonitrile eluant above this temperature). Sensitivity 0.2 AUFS for dicamba and 1.0 AUFS for remaining components.

(b) *Chromatographic column.*—250 × 4.6 (id) mm, Partisil 10 μm ODS-3 (Whatman Inc., 9 Bridewell Pl, Clifton, NJ 07014). Use with Whatman 50 × 4.6 (id) mm Co:Pell ODS pellicular guard column as in **6.276(b),** ref. 1. Flush column with water before and after regeneration with acetonitrile to obtain stable baseline.

(c) *Reaction vessel for sample and standard.*—50 mL conical glass centrifuge tube with leak-proof screw cap. 50 mL polypropylene centrifuge tube (Corning, Cat. No. 2533050) can be used for amine salt formulations but not for ester formulation due to absorption problem.

(d) *Container for prepared sample.*—1 oz narrow mouth linear polyethylene bottle (Cat. No. 2002-0001, Nalge Co., P.O. Box 365, Rochester, NY 16402, or equivalent).

Reagents

(a) *Sodium hydroxide solution.*—(1 + 1). Prepare as in **50.033(b),** ref. 1. Determine normality for use in preparing mobile phase.

(b) *Salicylic acid.*—Reagent grade (Aldrich Chemical Co., P.O. Box 2060, Milwaukee, WI 53201).

(c) *n-Butyrophenone.*—Reagent grade (Aldrich Chemical Co.).

(d) *Mobile phase.*—v/v with precise pH (2.68–2.70) control. Acetonitrile (distilled in glass)–HPLC quality water. Prepare water by filtering distilled deionized water through 0.45 μm filter. Transfer to 2 L Erylenmeyer flask, boil several minutes, and cap tightly with double layer of Al foil. Prepare 2 solvent systems (each pH 2.68–2.70) as shown:

	22% CH_3CN– 78% water	*33% CH_3CN– 67% water*
Water	725	610
Acetonitrile	220	330
NaOH solution (approx. 17.7N)	16.9	16.9
H_3PO_4 (concd), add to proper pH	31.8	33.0
	994	990

Add water for total volume of 1 L	6 / 1000 mL	10 / 1000 mL

Use graduated 25 mL pipet to add calculated volume of prepared NaOH solution for 0.3M final solution. Begin with approximately 10 mL short volume of water and after pH adjustment add balance of water for total volume of 1000 mL. Use standard buffer reference of pH 3.0 or 4.0 to standardize pH meter. Prepare mobile solvent one day in advance of assay.

(e) *Internal standard solution (ISS).*—Use isopropanol–water (2 + 1) as standard/sample solvent and diluent, and for rinsing when transferring 0.950 g salicylic acid into 1 L volumetric flask. Pipet in 9.0 mL butyrophenone, swirl, dilute to volume, and mix. Use salicyclic acid as reference for dicamba at 0.2 AUFS (peak height ca 95 mm) and butyrophenone for 2,4-D and MCPP at 1.0 AUFS (peak height ca 85 mm). Filter ISS as for sample.

Preparation of Standards and Sample

Dry standards 5–10 min at ≤85°C and weigh directly into reaction vessel according to ratio of herbicide percentages claimed. To keep peak heights on scale, largest component (percent) present, which is either 2,4-D or MCPP, must be used as governing factor. Target weight, for a recommended peak height of 115 mm at 1.0 AUFS or approximately 50% full scale deflection, is as follows: 2,4-D equivalent weight 0.150 g and MCPP equivalent weight 0.225 g. Because of variation in peak height response between detectors of different manufacture, each laboratory should prepare a test solution of known concentration to check detector response before proceeding with assay, and adjust sample size accordingly. Dicamba, always in least percent claim and correspondingly lower peak height response, can be prepared as separate standard for an optional procedure.

Final volume for all samples and standards is 25 mL. Weigh liquid sample, by difference, from dropping bottle into reaction vessel and pipet in 20 mL internal standard solution. Always use same pipet for samples and standards. To keep within 25 mL volume limit, sample volume greater than 5 mL requires evaporation from the reaction vessel in 65°C bath with air stream. Filter all samples and standards through 9.0 cm Whatman glass microfiber filter GF/C, or equivalent.

Determination

Flush column of previous solvent with water for several minutes before changing to mobile phase used in method. When baseline is stable, return effluent from detector (use Teflon tubing) back into solvent reservoir for recirculation of mobile phase, and cap flask with Al foil. An initial 10 μL injection of salicylic acid (0.95 g/L in isopropanol–water) (2 + 1) is recommended to condition column. For class I and II combination herbicides, use 22% acetonitrile–78% water first for early eluting components where dicamba is last component to elute in first solvent. Set sensitivity at 0.2 AUFS and inject 10 μL standard solution. After dicamba has eluted and pen returns to baseline, stop pump and switch Teflon tubing inlet (with filter) to 33% acetonitrile 67% water mobile phase. Flush system with syringe before starting pump again and set sensitivity at 1.0 AUFS. When instrument is equipped with 2 pumps, each pump may be dedicated to the specified mobile phase; this will reduce analysis time.

After changing to second mobile phase, initial component of interest to elute is 2,4-D, in about 5 min, which allows adequate equilibration time in second solvent. Second internal standard, butyrophenone, is required for second mobile phase. This neutral compound is unaffected by pH, i.e., only the strength of the solvent (acetonitrile) affects retention time. Injection sequence: standard, Sample 1, Sample 2, standard replicate, average standard peaks.

For class III herbicides (Table 2), use 33% acetonitrile–67% water eluant as an isocratic solvent.

As an optional procedure for class I and II combination formulations containing blends of dicamba and 2,4-D and with or without MCPP, use 33% acetonitrile–67% water eluant as an isocratic solvent and then repeat assay (replicate injection) using the first solvent 22% acetonitrile–78% water for dicamba. A key part of the alternative procedure is that when dicamba has eluted, remaining components may be flushed from the column with 50% acetonitrile–50% water (no buffer). Use separate dicamba standard with only salicylic acid ISS (0.950 g/L), thereby eliminating the delay in waiting for butyrophenone to elute, as in the method, before next injection. Do not change directly to 100% acetonitrile from buffered solvent because column will plug from salted-out phosphate buffer.

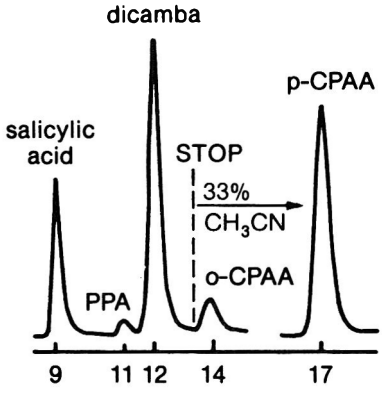

Figure 1. Separations of impurities from internal standard and dicamba in 22% CH_3CN.

Calculations

Calculations are based on automated integration of peak area or measurement of peak height, using the following formula:

$$\% = (R/R') \times (W'/W) \times \% \text{ purity of standard}$$

where R = sample ratio (herbicide)/internal standard and R' = same ratio of standard. W' = mg standard and W = mg sample.

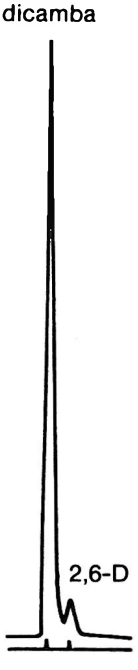

Figure 2. Separation of the 2,6-D isomer of 2,4-D from dicamba in 33% CH_3CN.

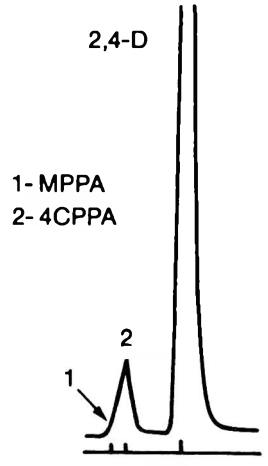

Figure 3. Separation of impurities from MCPP in 33% CH_3CN.

Discussion

Table 1 lists possible impurities plus 3 herbicides and 2 internal standards, a total of 28 solutes in a ternary combination formulation. Examination of elution order and retention times in Table 1 indicates that some impurities in the 33% acetonitrile solvent are not sufficiently resolved. Good resolution of early eluting impurities and peaks of interest is accomplished by improved column selectivity through the use of 22% acetonitrile as the first solvent. Of interest is the change in position of *o*-CPAA, in reference to the position indicated in Table 1. The chromatogram (Figure 1) is marked at the point where the pump is stopped and eluant is switched to 33% acetonitrile. Figure 2 shows the position of an

Figure 4. Separation of *p*-bromophenol from 2,4-D in 33% CH_3CN.

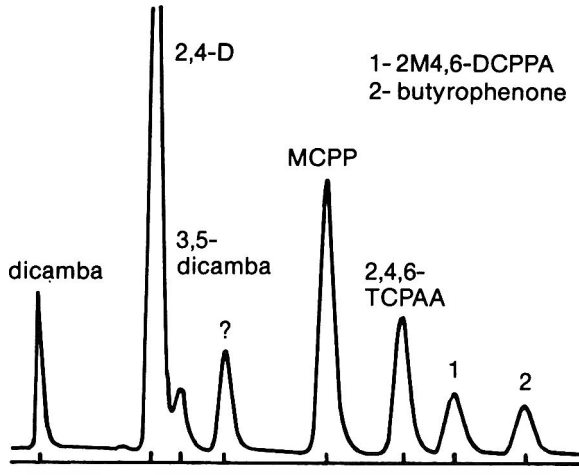

Figure 5. Separation of other impurities from 2,4-D, MCPP, and internal standard butyrophenone in 33% CH_3CN.

important impurity, the 2,6-D isomer from 2,4-D. The first impurities to appear after switching to the second solvent, 33% acetonitrile, are 2,6-D and *o*-CPAA which co-elute.

Both 2-MPPA and 4-CPPA are significant impurities from technical MCPP (Figure 3). As shown in Figure 4, *p*-bromophenol cannot be used as an internal standard because of co-elution with the impurities seen in Figure 3. In developing the automated HPLC method for 2,4-D (**6.275**), Skelly et al. (3) showed that the 2,6-dichlorophenol peak is positioned just before the 2,4-D peak. The same elution order of 2,4-D impurities is generally followed in both the column specified in the 2,4-D method and the ODS-3 column, with the difference that the latter column is more selective. We conclude that 2,6-dichlorophenol is resolved before the 2,4-D peak in this method, probably at the *p*-bromophenol position. Minor MCPP impurities, 2,6-DCPPA and 2-M-6-CPPA, are not resolved. Figure 5 shows separation of other impurities from 2,4-D, MCPP, and internal standard butyrophenone in 33% acetonitrile. Of these, 2-M-4,6-DCPPA is a significant impurity from MCPP. The unidentified peak is an impurity from 2,4-D, and corresponds to the unidentified peak in the chromatogram showing separation of impurity chlorophenoxy acids from internal standard and 2,4-D, which was reported in the 2,4-D method development paper of Skelly et al. (3).

In practice, butyrophenone has proven to be an excellent internal standard. Although it appears last in the chromatogram, there is relatively little zone spreading and virtually no peak tailing.

Two ternary formulations consisting of dicamba, 2,4-D, and MCPP were analyzed collaboratively in these laboratories, using a column which was 1 year old vs a new, unused column. Although there was a slight deterioration in resolution with the old column, quantitation was not affected.

Recommendation

The method, as described, has been shown to be capable of resolving the compounds of interest from all significant impurities. Therefore, its range of application covers single as well as ternary formulations containing mixtures of dicamba, 2,4-D, and MCPP in any combination. It is recommended that the method be subjected to collaborative study.

Acknowledgments

The authors are grateful to Timothy S. Stevens (Dow) and Charles Jones (Diamond Shamrock) for information received in consultation, and thank The Dow Chemical Co., Diamond Shamrock Corp., and Velsicol Chemical Corp. for supplying the reference compounds used in this study.

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DRUG RESIDUES IN ANIMAL TISSUES

Role of Anthranilic Acid in Background Levels of Sulfonamide in Porcine Livers when Determined by the Tishler Method

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Anthranilic acid occurs in excised swine livers as a result of temperature abuse before and/or after freezing. The tryptophan metabolite is the major source of the variable non-sulfonamide background level frequently encountered in the Tishler method for determining sulfonamide residues in swine livers. Diazotized anthranilic acid reacts slowly with *N*-1-(naphthyl)ethylenediamine [$k(s^{-1}) = 5.79\ (\pm 0.07) \times 10^{-5}$] and the final background level depends on the time elapsed between addition of the coupling agent and spectrophotometric determination. Kynurenine was tentatively identified as a minor source of the non-sulfonamide background level.

The procedure of Tishler et al. (1) (commonly referred to as the Tishler method), specifying the Bratton–Marshall (2) reaction, is the unofficial method of choice used by the regulatory agencies for determining sulfonamide residues in edible animal tissues. The colorimetric procedure, based on the coupling of *N*-1-(naphthyl)ethylenediamine to diazotized primary aromatic amines, is not specific for sulfonamides; therefore, the procedure is potentially subject to erroneous results. It is not unusual to conclude, on the basis of results of the Tishler method, apparent sulfonamide residues of 0.01–0.05 ppm in liver tissues obtained from animals maintained in carefully controlled sulfonamide-free environments (3). Furthermore, it is well known that higher values can occur if the tissues are subjected to temperature abuse before analysis (3). Temperature abuse may be responsible, at least partially, for wide variations in results on the same samples assayed by different laboratories (4).

In a recent publication (5), we identified anthranilic acid (*o*-aminobenzoic acid), a naturally occurring Bratton–Marshall-positive compound, in swine liver extracts analyzed by a slight modification of the Tishler method. The present manuscript defines the role of anthranilic acid in the variable "background level" of the Bratton-Marshall Tishler method for sulfonamide residues in swine livers and presents other observations made during the course of the study.

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Experimental

Reagents

(a) *Solvents.*—Acetone, hexane (Mallinckrodt, Inc., St. Louis, MO), and ethyl acetate (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442) were distilled in glass; chloroform, Baker Analyzed reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08665).

(b) *Chemicals.*—Anthranilic acid (Eastman Kodak, Rochester, NY (14650); kynurenine (Sigma Chemical Co., St. Louis, MO 63178); *o*-aminoacetophenone (Aldrich Chemical Co., Milwaukee, WI 53233).

(c) *N-1-(Naphthyl)ethylenediamine (NEDA) dihydrochloride.*—0.1% aqueous solution (Sigma Chemical Co.).

(d) *Sodium nitrite.*—0.1% aqueous solution.

(e) *Ammonium sulfamate.*—0.5% aqueous solution.

Apparatus

(a) *Tissue grinder.*—Brinkmann Polytron® homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590).

(b) *Centrifuge.*—Servall Superspeed centrifuge, type SS-1 Rotor (Ivan Sorvall, Inc., Norwalk, CT).

(c) *Vortex stirrer.*—Super Mixer (Lab-Line Instruments, Inc., Melrose Park, IL 60160).

(d) *Spectrophotometer.*—Hitachi-Perkin Elmer Model 139 UV-visible spectrophotometer operated at 545 nm.

(e) *Thin layer chromatographic apparatus and reagents.*—2.5 × 10 cm precoated (250 μm) silica gel G glass plates (Analtech, Inc., Newark, DE 19711). Anthranilic acid solvent system: ethyl acetate–methanol (4 + 1); *o*-aminoacetophenone solvent system: hexane–ethyl acetate (4 + 1).

(f) *UV source.*—Chromato-Vue (Ultra-Violet Products, Inc., San Gabriel, CA 91778).

Liver Samples

Swine livers were obtained from a local slaughter house immediately after slaughter. After being excised from the animals, 4 adjacent portions (25–30 g) were sliced from a section of the organ. One portion (zero abuse) was frozen immediately in crushed dry ice and the others were held 1, 3, and 5 h, respectively, at ambient temperature before freezing at −15°C.

Modified Bratton-Marshall Tishler Method (BMTM)

Fifteen mL chloroform–acetone (1 + 1) was added to 5 g partially thawed tissue in a 50 mL polypropylene centrifuge tube and the mixture was homogenized at low speed for 1 min. The homogenate was centrifuged 2 min at 3500 rpm. The solvent was removed with a disposable Pasteur pipet and filtered through glass wool packed in a super Pasteur pipet. The filtrate was collected in a 50 mL polypropylene screw-cap centrifuge tube. The tissue was re-homogenized with additional 15 mL chloroform–acetone (1 + 1) and centrifuged, and the solvent was recovered and filtered through glass wool into the 50 mL screw-cap centrifuge tube. The combined extracts were evaporated just to dryness under a stream of nitrogen at 50°C. The residue was dissolved in 0.6 mL acetone and 15 mL hexane with vortex mixing. The solvent was extracted with two 1.25 mL portions of 1N HCl in a rocking motion for 3 min followed by centrifugation at 3500 rpm for 2 min. The aqueous layers were recovered with a disposable Pasteur pipet and filtered through glass wool packed in a Pasteur pipet into a 10 mL graduated cylinder. The entire procedure was repeated on a second 5 g sample, and the HCl extracts from each sample were combined, diluted to 6 mL with 1N HCl, mixed thoroughly, and divided into two 3 mL portions in 9 mL screw-cap specimen vials. To each sample and a 3 mL blank sample of 1N HCl, 0.2 mL 0.1% aqueous sodium nitrite was added. The solutions were mixed thoroughly, allowed to stand for 3 min, 0.2 mL 0.5% aqueous ammonium sulfamate was added, and solutions mixed thoroughly and allowed to stand for 2 min. To one sample and the HCl blank, 0.2 mL 0.1% aqueous NEDA was added; to the remaining sample (control), 0.2 mL water was added. Solutions were mixed thoroughly and allowed to stand in the dark. Absorbances were determined at 545 nm in a 1 cm cell at 0.25, 1.0, 2.0, and 4.0 h after addition of NEDA.

Anthranilic Acid – BM Reaction

To determine reaction rate, 2.64 μg anthranilic acid/3 mL 1N HCl was subjected to the BM reaction according to the procedure outlined above. Absorbances were obtained at 545 nm (maximum absorption, 550 nm) in 1 cm cell at various intervals following addition of NEDA.

Absorptivities at 1, 2, and 4 h reaction times were obtained on standard solutions of 0.22–3.08 μg anthranilic acid/3 mL 1N HCl.

Results and Discussion

Preliminary studies on numerous swine livers, using the previously described (6) thin layer chromatographic (TLC) screening procedure for sulfamethazine and sulfathiazole, demonstrated that the majority of samples frozen in dry ice immediately after being excised from the animal contained only trace amounts of anthranilic acid. However, if the livers were abused, that is, held at ambient temperatures before freezing, the concentration of the metabolite increased significantly in about half of the livers analyzed, and the concentration increased with increased time of abuse. The same results were observed by TLC of methanol effluents from Chromosorb 102 which was shown previously (5) to adsorb anthranilic acid from liver extracts obtained by the Tishler method. Based on these initial observations, the actual contribution of anthranilic acid to the variable background levels observed in the Tishler method for determining sulfonamide residues in swine livers was investigated.

Studies on anthranilic acid in the Bratton-Marshall reaction revealed that, in contrast to diazotized sulfonamide which reacts in 15 min, diazotized anthranilic acid reacted more slowly with the coupling agent (NEDA). The reaction for a series of known concentrations of anthranilic acid was complete, for practical purposes, within 24 h, with a mean absorptivity of 230 (L/g/cm light path). Hence by using the absorptivity equation ($E = A/(c \times l)$, where E = absorptivity; A = absorbance; c = concentration in g/L; and l = length of light path in cm), it is possible to determine the amount of diazotized anthranilic acid in a standard solution reacted at time t. An analysis of results obtained on a known concentration of anthranilic acid showed the reaction to be first-order and could be expressed mathematically by $kt = \ln a^\circ/a$ (k = rate constant, a° = initial concentration, and a = concentration remaining at time t). Using this equation, the slope of a straight line, determined by the method of least squares, corresponding to

the reaction rate [$k(s^{-1}) = 5.79\ (\pm 0.07) \times 10^{-5}$] was obtained (0.07 = mean deviation of duplicate samples) ($r = 0.9997$). The reaction was calculated to be 95% complete in 14.4 h. Spectrophotometric studies on liver extracts spiked with anthranilic acid demonstrated, however, that holding samples for extended periods before spectrophotometric determination, in addition to being impractical, resulted in the development of a non-Bratton–Marshall blue color and an occasional cloudy reaction mixture. To overcome these limitations and still obtain quantitative anthranilic acid data, mean absorptivity values of known concentrations of anthranilic acid in the reaction were determined at 1 ($E = 46$), 2 ($E = 83$), and 4 h ($E = 136$) reaction times.

Table 1 compares the results (in absorbance units) of 2 samples of swine livers held at ambient temperature for 0, 1, 3, and 5 h before freezing and analyzed by the modified Tishler method. Absorbances were determined after 0.25, 1.0, 2.0, and 4.0 h following addition of NEDA. In most samples analyzed, trace amounts of sulfamethazine were observed by the TLC screening procedure which may account for all or a portion of the absorbance of non-abused samples read after 0.25 h color development. As shown by both TLC and spectrophotometric determination, liver A developed very little anthranilic acid, even in the sample abused for 5 h. In contrast, liver B developed significant amounts, the concentration of which increased with increasing time of abuse. As important to the final results is the increase in absorbance as the time differential between addition of NEDA and spectrophotometric determination increased. For example, the 5 h abused liver B read 0.25 h after addition of NEDA had an absorbance of 0.009, equivalent to 0.034 ppm sulfamethazine (0.1 ppm sulfamethazine, the regulatory violative level, in 5 g liver has an absorbance of 0.026). However, if the reaction was allowed to proceed for 1 h, the maximum time recommended (3), 0.077 ppm apparent sulfonamide (based on sulfamethazine) would be concluded based on the absorbance of 0.020, but actually a maximum of 0.011 ppm (based on non-abuse, 0.25 h absorbance) is present in the sample.

Applying the absorptivity values determined at 1, 2, and 4 h reaction time to the absorbances obtained on liver B (minus non-abuse, 0.25 h absorbance) suggested that small amounts of other non-sulfonamide Bratton–Marshall-positive compounds were also present in the 3 and 5 h abused samples. TLC on extracts obtained by the screening procedure from abused livers with

Table 1. Effect (measured in absorbance unit) of abuse and elapsed time before spectrophotometric determination on results for sulfonamide residues in swine livers

Abuse time, h[a]	Absorbance time, h[b]			
	0.25	1.0	2.0	4.0
	Liver Sample A			
0	0.002	0.002	0.002	0.002
1	0.002	0.004	0.005	0.007
3	0.004	0.007	0.009	0.011
5	0.004	0.007	0.009	0.011
	Liver Sample B			
0	0.003	0.004	0.006	0.009
1	0.003	0.006	0.010	0.013
3	0.005	0.011	0.016	0.021
5	0.009	0.020	0.032	0.049

[a] Time fresh swine liver held at ambient temperature before freezing.

[b] Hours following addition of NEDA reagent.

significant amounts of anthranilic acid revealed trace amounts of an unknown positive compound at the origin following solvent development. The unknown was tentatively identified as kynurenine on the basis of the odor, fluorescence, positive Bratton-Marshall reaction, and TLC characteristics of its alkaline hydrolysis product *o*-aminoacetophenone (7). Kynurenine is not considered a major factor in the Tishler method because of its low solubility in organic solvents and a molar absorptivity of 8500 in the Bratton-Marshall reaction (ϵ sulfamethazine = 52 500). It is estimated that kynurenine contributed less than 0.01 ppm to 5 h abused liver B sample reported in Table 1.

To determine the effect of frozen storage on the development of anthranilic acid in swine liver, fresh samples were obtained and each sample was divided into 2 portions. One portion of each sample was abused before freezing. The second portion of each sample was frozen immediately and held at −15°C for 2–4 months before being thawed and abused. Typical results of these studies are presented in Table 2. Anthranilic acid did not develop during frozen storage. However, temperature abuse after freezing resulted in higher concentrations of anthranilic acid compared with abuse before freezing. This suggests that freezing and thawing significantly disrupts liver cells, thereby increasing the substrate (i.e., tryptophan or its metabolites) available for enzymatic degradation to anthranilic acid. The variability in individual

Table 2. Effect of swine liver abuse on formation of anthranilic acid (ppm) [a]

Liver sample	Abuse treatment	Abuse time, h [b]			
		0	1	3	5
C	before freezing	0.03	0.03	0.05	0.04
	after freezing	0.03	0.04	0.08	0.09
D	before freezing	0.02	0.04	0.12	0.19
	after freezing	0.03	0.05	0.16	0.28

[a] Based on 4 h absorbances (minus zero h abuse, 0.25 h reading).
[b] Time swine liver was held at ambient temperature.

Table 3. Effects on abuse treatments before and after freezing on formation of anthranilic acid in swine liver

Abuse time, h [a]			
Before freezing	After freezing	Total	Anthranilic acid, ppm [b]
0	0	0	0.04
1	0	1	0.05
3	0	3	0.22
5	0	5	0.30
0	0	0	0.05
0	1	1	0.13
1	2	3	0.28
3	2	5	0.50

[a] Time swine liver was held at ambient temperature.
[b] Based on 4 h absorbances (minus zero h abuse, 0.25 h reading).

liver samples to develop significant amounts of anthranilic acid is again evident.

Table 3 demonstrates the additive effect of abusing samples before freezing and following frozen storage. Liver samples which had been previously abused 0, 1, and 3 h at ambient temperature before freezing were thawed after 1 month storage at −15°C and abused at ambient temperature for an additional 1, 2, and 2 h, respectively. As might be expected, anthranilic acid concentration increased in the doubly abused samples relative to samples abused only before freezing for the corresponding length of time.

The results of these studies establish anthranilic acid as the major source of the variable non-sulfonamide Bratton-Marshall background levels encountered in the Tishler method for sulfonamide residues in swine livers. The variability in levels depends on the availability of precursor and/or the ability of individual livers to develop anthranilic acid as a result of temperature abuse before and/or after freezing, the length of temperature abuse, and the time between addition of NEDA to diazotized samples and spectrophotometric determination. Hence, it is erroneous to arbitrarily subtract a fixed background level from unknown liver samples as has been done in the past (3, 4) by regulatory agencies and research institutions. The need for a quantitative procedure free of background interferences is evident. Until such procedures are developed, increased care must be taken in sample handling, and the time of spectrophotometric determination must be standardized to 0.25 h following addition of NEDA to ensure that accurate and reproducible results are obtained by the Tishler method and similar Bratton-Marshall procedures.

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Modified Method for Electron Capture Gas-Liquid Chromatographic Determination of Diethylstilbestrol Residues in Urine of Fattened Bulls

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A gas-liquid chromatographic (GLC) method with electron capture (EC) detection was developed for determining diethylstilbestrol residues in the urine of fattened bulls. Diethylstilbestrol (DES) is extracted into benzene, and then into 1N sodium hydroxide. The pH of the phenolic fraction (alkaline phase) is adjusted to 10.2 and DES is extracted again into benzene. Sample extracts are cleaned up on silica gel. Trifluoroacetic anhydride (TFAA) is used as acylation reagent, and the derivatized sample is chromatographed on a 3% OV-17 column and measured with a ^{63}Ni EC detector. The method is suitable for determining residues at levels as low as 2 ppb.

The synthetic estrogen diethylstilbestrol (DES) has gained importance as a human therapeutic drug, and is used commercially as an aid in fattening cattle for slaughter. As a growth-promoting agent, DES is administered either as a surgical implant in pellet form or as a feed additive. Because of its estrogenic and carcinogenic properties, DES residues are not permitted in food of animal origin (zero tolerance). However, DES is still used illegally in animal husbandry in the United States and Europe (1, 2). We needed a method to determine whether DES is used in Greece.

Several procedures have been developed for measuring DES in animal tissues (3–5), but because some authors have emphasized the importance of urine and fecal excreta (6–8), we decided to detect DES in urine samples.

We carried out a study based on published procedures (9–11). The method has been successfully used in our laboratory for the determination of DES residues at levels as low as 2 ppb in the urine of fattened bulls.

METHODS

Apparatus

(a) *Glassware.*—All glassware was immersed in 10% nitric acid 1 h, rinsed thoroughly with distilled water, and dried at 110°C.

(b) *Gas chromatograph.*—Varian Model 3700, with ^{63}Ni electron capture detector, 200 cm × 6.35 mm × 2 mm id glass column packed with 3% OV-17 on 80–100 mesh Chromosorb W(HP). Operating conditions: nitrogen carrier gas, 99.997% pure, 30 mL/min; temperatures (°C)—injector 210, detector 250, column 180. Attenuation 32.

(c) *Chromatographic column.*—10 × 100 mm Quickfit with 50 mL reservoir packed with silica gel 60, 0.063–0.2 mm, 70–230 mesh ASTM (Merck No. 7734) in benzene–ethyl acetate (85 + 15 v/v).

Reagents

(a) *Solvents.*—Benzene. (Caution: Benzene is a possible carcinogen. No other satisfactory solvent has been found.)

(b) *Trifluoroacetic anhydride (TFAA).*—Test purity by evaporating 1 mL TFAA (Merck) to dryness in 15 mL centrifuge tube equipped with Teflon-lined cap. Dissolve residue in 2 mL distilled *n*-hexane and inject exactly 2 μL into gas chromatograph under conditions used for measuring diethylstilbestrol. If interfering substances are present, distill TFAA in all-glass system equipped with drying tube. Discard first 10% and last 20% of distillate. Test collected TFAA as described.

(c) *DES stock solution.*—1000 ppm. Dissolve 100 mg *trans*-DES and dilute to 100 mL with acetone. Let stand 24 h before use. Store in dark; prepare fresh monthly.

(d) *DES working solution.*—1 ppm. Prepare by diluting DES stock solution. Store in dark; prepare fresh daily.

Preparation of Sample

Before analysis, let urine samples stand 24 h at room temperature (ca 20°C) for hydrolysis of DES conjugates. If samples have been frozen (−15°C), thaw and let stand 18 h at 22°C.

Extraction

Filter 50 mL urine and make strongly acid (ca pH 1). Transfer sample to 250 mL separatory

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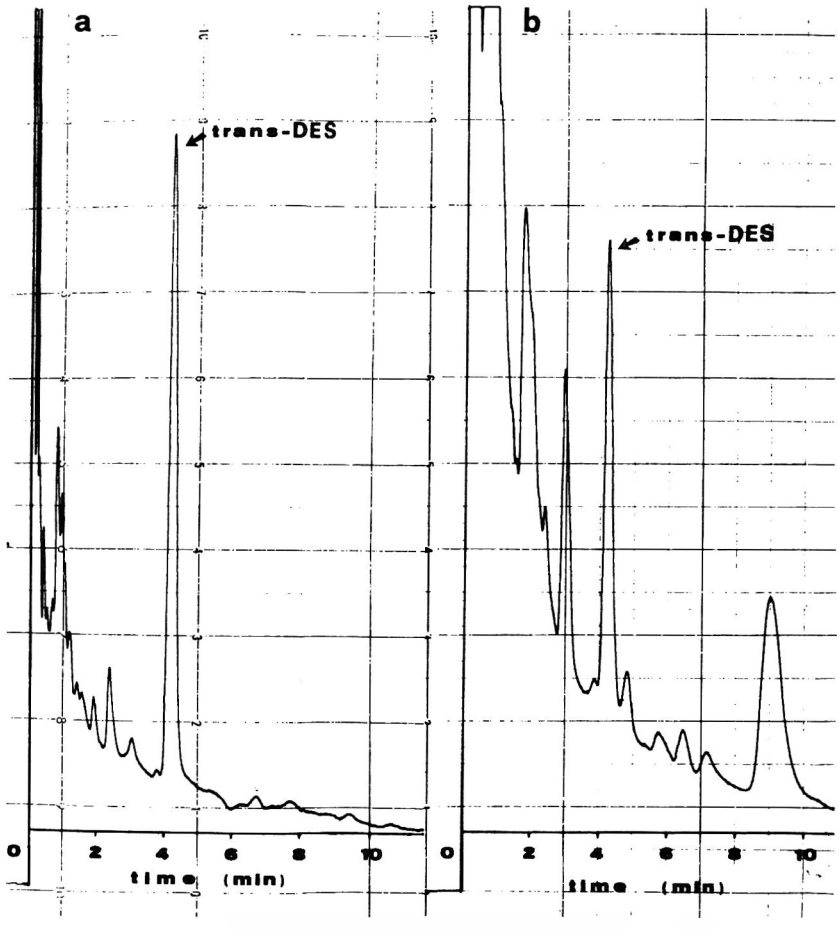

Figure 1. Gas chromatograms of (a) *trans*-DES-TFA standard and (b) *trans*-DES-TFA in urine sample from fattened bull.

funnel and extract with two 50 mL portions of benzene. Let layers separate and transfer benzene layer into another 250 mL separatory funnel. Extract combine benzene layers with two 50 mL portions of 1N NaOH. Let layers separate, and transfer alkaline phase to 250 mL beaker. Adjust pH of combined phenolic fractions to 10.2 and extract again with two 50 mL portions of benzene. After layers separate, discard lower layer and transfer benzene extracts to 250 mL round-bottom flask through 3–4 cm high column of anhydrous Na_2SO_4. Wash column with five 10 mL portions of benzene and collect eluate in same flask.

Column Chromatography

Evaporate combined benzene extracts to dryness by using 40°C water bath and vacuum rotary evaporator. Dissolve residue in 1 mL benzene–ethyl acetate (85 + 15 v/v). Pour silica gel in suspension with benzene–ethyl acetate into 10 × 100 mm glass column fitted with coarse porosity fritted glass filter to depth of 9 cm. Wash column with 20 mL of above mixture. Transfer residue to top of column by using another 1 mL benzene–ethyl acetate mixture to complete transfer. Elute with further 10 mL of the same mixture at a maximum flow rate of 1.5 mL/min (this eluate contains the DES residue). Transfer this eluate to a 15 mL centrifuge tube equipped with Teflon-lined cap by using 2–3 mL benzene–ethyl acetate.

Derivatization

Perform analysis on sample and standard solution at same time.

Transfer 1 mL working standard solution to another centrifuge tube. Evaporate sample and

standard to dryness in 40°C water bath under gentle stream of nitrogen. Add to each tube 1 mL TFAA, stopper tightly, and let stand 2 h at room temperature (ca 20°C). Evaporate to dryness under gentle stream of nitrogen, and redissolve residue in 2 mL distilled *n*-hexane.

Gas-Liquid Chromatography

Inject 2 μL derivatized standard into the chromatograph to determine whether derivatization has been successful. Then inject 2 μL derivatized sample and compare area only of *trans*-DES-TFA peak with that produced from standard. Inject standard from time to time to compensate for slight changes in instrument response (Figure 1).

Calculation

Use peak height only of *trans*-DES-TFA peak to determine ppb DES in sample by measuring peak height width at one-half height.

Results and Discussion

Sixty urine samples from fattened bulls (not steers) were analyzed by the described method. Diethylstilbestrol was detected in 10 samples (16.7% positive). Concentrations ranged between 8 and 40 ppb. Presence of DES in these samples was confirmed by the thin layer chromatographic (TLC) method of Vogt (12).

Metabolic studies on DES in both ruminants and nonruminants indicated that DES occurs in both urine and bile as the monoglucuronide (13). Other studies showed that the major portion of the DES occurs in urine as either DES or as a hydrolyzable conjugate of DES (8). It is also noteworthy that the proportion of conjugate in urine decreased with storage and handling, indicating a gradual hydrolysis to the free form. Examinations of fresh urine samples indicated that a high proportion (perhaps all) of the DES was present in conjugated form at the time of excretion (14). Others observed that putrefaction in urine converts the conjugate to the free form (15).

Because hydrolysis of DES-glucuronide is usually effected to facilitate later purification steps, HCl hydrolysis was used and showed that this procedure did not release significant amounts of free DES, and more vigorous conditions, such as boiling in aqueous HCl, caused low recoveries of free DES (16). Ryan (17) stated that acid hydrolysis of DES-glucuronide can give low DES recoveries by being too destructive, and that enzyme hydrolysis can give low recoveries by being too specific.

This entire question was further studied in a series of papers from the U.S. Department of Agriculture, which showed that DES could be freed from conjugates in urine samples on standing at room temperature. According to these studies, the nature of the DES in urine depended on the history of the sample before analysis. The majority of the DES was obtained if the urine had been allowed to accumulate for 12 h and then stored at −15°C. When these urine samples were thawed and held 18 h at 22°C before chromatography, most of the DES was characterized by TLC as free DES. This proportion appeared to increase when urine had been allowed to stand for 24 h before cooling (7). Therefore, we decided to deconjugate DES-G by standing at room temperature.

This entire area needs further experimentation on control urine "spiked" with DES-G to determine the degree of recovery of free DES. Such trials are being developed.

Estrogens in general and DES in particular are phenols. Because of this characteristic property, they can be partitioned between organic and aqueous phases by choosing a suitable pH. We noted that at pH 10.2, a higher percentage of DES was recovered.

Having observed that the *trans*-DES peak was predominant and the *cis*-DES peak was always small and of stable height (± 10%), we used only the *trans*-DES isomer for calculation.

Following this procedure, the recovery of the entire technique is approximately 81%. An analyst trained in this method can perform 5–10 analyses a day, depending on the level of detection desired.

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FERTILIZERS

Comparison of AOAC and Atomic Absorption Spectrophotometric Methods for Determining Sodium in Fertilizers: Collaborative Study

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A collaborative study was carried out to compare AOAC method 2.147-2.150 for determining sodium in fertilizers with the atomic absorption spectrophotometric (AAS) method. Twelve synthetic NPK samples, 6 pairs of blind duplicates (0.06-8.5% Na), were sent to 22 collaborators; a total of 13 sets of results were received and evaluated by the modified matched pairs technique. The AAS method showed better performance in samples with low sodium content and high potassium content. The AAS method has been adopted official first action for determining sodium in fertilizers.

The official first action method, **2.147-2.150,** (1) based on a flame emission spectrophotometric (FES) procedure was compared in 2 previous studies (2, 3) with the atomic absorption spectrophotometric (AAS) procedure and a sodium selective ion electrode (SSE) method. Results from these studies showed that the AAS method could perform better than the FES method. Furthermore, according to Magruder Reports (4-9), it appears that the AAS method is used more frequently than the FES method. Therefore, a collaborative study was carried out to evaluate the performance of the AAS method and the FES method in different laboratories.

Collaborative Study

Six fertilizer samples were prepared and each was divided into 2 parts to distribute as blind duplicates. The 12 resulting samples (Table 1) were sent to 22 collaborators. Each collaborator was supplied with a copy of the 2 methods and was instructed to: (*1*) practice each method once on any sample; (2) analyze each sample once by each method, following the analytical procedures exactly; (3) analyze Samples 1 to 6 one day, and Samples 7 to 12 another day; (4) have one analyst perform all determinations; (5) report any comment on the performance of the methods.

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The recommendations of the Associate Referee were approved by the General Referee and Committee A and were adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

METHODS

The flame emission spectrophotometric (FES) method was used as shown in **2.147-2.150** (1).

Sodium in Fertilizers

Atomic Absorption Spectrophotometric Method

Official First Action

2.D19 ***Reagents and Apparatus***

(**a**) *Ammonium oxalate soln.*—Dissolve 40 g $(NH_4)_2C_2O_4$ in 1 L H_2O.

(**b**) *Sodium chloride.*—Dry 2 h at 105°.

(**c**) *Atomic absorption spectrophotometer.*—Model AA6 (Varian Techtron Pty Ltd, 679-701 Springvale Rd, Mulgrave, Vic., Australia 3170), or equiv.

2.D20 ***Preparation of Solution***

Weigh 2.5 g (<4% Na) or 1.25 g (4-20% Na) sample into 250 mL vol. flask, add 125 mL H_2O and 50 mL $(NH_4)_2C_2O_4$ soln, and boil 30 min. Cool, dil. to vol., mix, and pass thru dry filter. For samples contg <1% Na, use this soln for detn. For samples contg 1-20% Na, place 20 mL in 100 mL vol. flask, dil. to vol. with H_2O, and mix.

2.D21 ***Preparation of Standard Curve***

Dissolve 2.5421 g dried NaCl in H_2O and dil.

Table 1. Description of blind duplicate samples used in collaborative study

Samples	Description	Approx. Na content, %
1 and 7	20–10–35, mixture of diammonium phosphate, potassium chloride, ammonium chloride, and sodium chloride	0.06
2 and 8	10–25–33, mixture of diammonium phosphate, potassium chloride, and sodium chloride	0.10
3 and 9	10–10–0, mixture of ammonium sulfate, normal superphosphate,[a] and sodium chloride	2.3
4 and 10	7–7–15, mixture of ammonium sulfate,[a] normal superphosphate,[a] potassium chloride,[a] and sodium chloride	2.6
5 and 11	10–10–20, mixture of diammonium phosphate,[a] potassium chloride, ammonium chloride, and sodium chloride	8.0
6 and 12	6–31–0, mixture of ammonium sulfate,[a] normal superphosphate,[a] and monosodium phosphate	8.5

[a] Fertilizer grade product. Others are reagent grade products.

to 1 L (1000 ppm Na). Prep. std solns to cover range 0–200 ppm at intervals ≤20 ppm Na.

2.D22 ***Determination***

Set wavelength at 330.3 nm using air–C_2H_2 flame. Aspirate stds and samples. Plot curve from std values and det. Na content of sample solns from plot of A against ppm Na. Calc. % Na as follows:

$$\leq 1\%: \quad \text{ppm Na} \times 25/M = \%\text{Na}$$

$$1\text{–}20\%: \quad \text{ppm Na} \times 125/M = \%\text{Na}$$

where M = wt of sample (mg).

Results and Discussion

A total of 13 complete sets of results were received from collaborators. All individual collaborative results with averages, standard deviations, and coefficients of variation for each sample are shown in Tables 2 and 3.

The sets of results were analyzed by the 2-sample X-Y chart (10), which is not included here, forming 6 unit blocks for each method pairing Sample 1 with Sample 2, Sample 3 with Sample 4, etc. As usual, the points were found predominantly in quadrants I and III, representing +, + and −, − results, and with some exceptions were also generally clustered near the average concentrations in an elliptical pattern around a major axis at 45°.

The results of applying the ranking test described by Youden (10) are presented in Tables 4 and 5. For the FES method, Collaborator 3 had a total rank of 41.5, which is outside the 95% confidence limits, and therefore was not included in further statistical analysis because of the consistently low bias. For the AAS method, using the same criteria, Collaborator 3, with a total rank of 30, and Collaborator 11, with a score of 133.5, were eliminated because of their low and high bias, respectively.

Dixon's test for outlier individual results (11) was applied to remaining values with a 1 in 20 probability of a wrong decision, and 8 additional values for the FES method and 5 more values for the AAS method were rejected. Some additional values for both methods were not considered in calculations because they were paired with outlier results.

After applying rejection tests, the remaining individual results were analyzed by Wernimont's variation to the Youden's matched pairs technique (10). Within days (σ_{WD}), from one day to another ($\sigma_{D/L}$), from one laboratory to another (σ_L), and total (σ_T) error estimates are shown in Table 6. Results of applying the F-test for sets of samples are shown in Table 7, and results of the t-test for each sample are presented in Table 8.

When estimates of σ^2_{WD}, $\sigma^2_{D/L}$, σ^2_L, and σ^2_T were compared by means of the F-test (Table 7), 5 values were significantly different at the 95% level of confidence and 4 were in favor of the AAS method.

It is important to note that the greatest differences found were for Set I (low sodium content), and this was the only set where significant differences for σ^2_T were found. This indicates that the AAS method has better reproducibility at a low sodium level than does the FES method.

Coefficients of variation (Table 8), are low for the 2 methods with the exception of Samples 1, 2, 7, and 8 which were low in sodium content.

Sodium averages obtained for each sample were compared by the t-test at the 95% level (Table 8), and significant differences were found in the averages for Samples 1, 2, 7, and 8 (low sodium content). The other samples did not show significant differences.

Table 2. Collaborative results (%) for determination of sodium in fertilizers by FES method

	Sample											
Coll.	1	2	3	4	5	6	7	8	9	10	11	12
1	0.095	0.152	2.221	2.929	7.755	8.389	0.102	0.149	2.328	2.997	8.021	8.688
2	0.098	0.140	2.490	3.230	8.110	8.820	0.092	0.140	2.210	3.080	7.550	8.510
3	0.110[a]	0.144[a]	2.180[a]	2.880[a]	7.850[a]	8.450[a]	0.114[a]	0.152[a]	2.180[a]	2.830[a]	7.850[a]	8.350[a]
4	0.121	0.170	2.300	2.990	7.700	8.680	0.110	0.160	2.360	3.020	8.040	8.740
5	0.180[b]	0.210[a]	2.250[b]	2.500[a]	7.800	8.400	0.150[b]	0.200[b]	2.600[b]	3.380[a]	8.300	8.850
6	0.140	0.170	2.400	2.950	7.900	8.100	0.120	0.150	2.250	2.875	7.750	8.500
7	0.124	0.169	2.267	2.957	7.923	8.695	0.122	0.152	2.255	3.001	7.953	8.562
8	0.141	0.177	2.421	2.955	7.874	8.750	0.126	0.166	2.265	2.839	8.072	8.691
9	0.120	0.155	2.350	2.940	7.850	8.750	0.128	0.172	2.480	2.910	7.900	8.700
10	0.142	0.168	1.910[a]	2.760[b]	7.750	8.625	0.132	0.169	1.926[a]	2.322[a]	7.750	8.375
11	0.103	0.144	2.579	3.283	8.660[a]	9.181[b]	0.119	0.156	2.461	2.995	8.512[b]	9.772[a]
12	0.130	0.153	2.350	3.050	8.000	8.880	0.115	0.147	2.280	2.950	7.630	8.500
13	0.120	0.170	2.280	2.950	8.000	8.800	0.125	0.182	2.300	2.900	7.750	8.700
Av.	0.1213	0.1607	2.3658	3.0234	7.8784	8.6263	0.1174	0.1585	2.3189	2.9567	7.8833	8.6196
Std dev.	0.0168	0.0123	0.1096	0.1281	0.1250	0.2355	0.0120	0.0126	0.0903	0.0747	0.2206	0.1393
Coeff. of var., %	13.85	7.65	4.63	4.24	1.59	2.73	10.22	7.95	3.89	2.53	2.80	1.62

[a] Values were rejected. See text.
[b] Values not considered in computations because they are paired with outlier values.

Table 3. Collaborative results (%) for determination of sodium in fertilizers by AAS method[a]

	Sample											
Coll.	1	2	3	4	5	6	7	8	9	10	11	12
1	0.100	0.132	2.423	2.989	7.854	8.587	0.097	0.130	2.392	3.021	8.021	8.588
2	0.085	0.120	2.120	2.800	7.980	8.880	0.081	0.120	2.160	2.740	7.650	8.640
3	0.074[a]	0.104[a]	2.190[a]	2.770[a]	7.390[a]	8.120[a]	0.077[a]	0.106[a]	2.180[a]	2.790[a]	7.490[a]	8.090[a]
4	0.150[a]	0.180[b]	2.240	2.850	8.020	8.810	0.120[b]	0.150[b]	2.250	2.930	7.710	8.550
5	0.070	0.090	2.070	3.100	7.600[b]	7.900[a]	0.120	0.130	2.500	2.860	7.000[a]	7.600[a]
6	0.100	0.130	2.160	2.850	7.800	8.100	0.100	0.120	2.160	2.750	7.900	8.000
7	0.094	0.129	2.352	3.007	7.955	8.668	0.092	0.127	2.291	2.951	7.954	8.545
8	0.088	0.122	2.436	2.969	7.660	8.541	0.114	0.144	2.294	2.801	7.810	8.521
9	0.100	0.130	2.324	3.000	7.900	8.740	0.096	0.140	2.420	2.962	8.100	8.700
10	0.127	0.156	2.090[b]	2.586[a]	7.660	8.721	0.118	0.150	2.080[b]	2.470[b]	7.800	8.480
11	0.111[a]	0.146[a]	2.371[a]	3.059[a]	8.090[a]	8.776[a]	0.118[a]	0.149[a]	2.362[a]	3.043[a]	8.527[a]	8.694[a]
12	0.095	0.125	2.330	2.950	7.750	8.650	0.100	0.130	2.300	2.930	7.550	8.300
13	0.100	0.160	2.370	2.980	8.200	8.800	0.100	0.130	2.370	2.920	8.000	8.750
Av.	0.0959	0.1294	2.2825	2.9495	7.8779	8.6497	0.1018	0.1321	2.3137	2.8865	7.8495	8.5074
Std. dev.	0.0145	0.0193	0.1283	0.0939	0.1696	0.2193	0.0122	0.0098	0.1094	0.0948	0.1768	0.2172
Coeff. of var., %	15.12	14.91	5.62	3.18	2.15	2.54	11.98	7.42	4.73	3.28	2.25	2.55

[a] Values were rejected. See text.
[b] Values not considered in computations because they are paired with outlier values.

Table 4. Ranked collaborator results for FES method

Coll	Sample 1	2	3	4	5	6	7	8	9	10	11	12	Total rank
1	1	4	3	4	3	2	2	3	9	9	9	7	56
2	2	1	12	12	12	11	1	1	3	12	1	5	73
3	4	2.5	2	3	5.5	4	4	5.5	2	2	6	1	41.5[a]
4	7	10	7	10	1	6	3	8	10	11	10	11	94
5	13	13	4	1	4	3	13	13	13	13	12	12	114
6	10	10	10	6.5	8	1	7	4	4	4	4	3.5	72
7	8	8	5	9	9	7	8	5.5	5	10	8	6	88.5
8	11	12	11	8	7	8.5	10	9	6	3	11	8	104.5
9	5.5	6	8.5	5	5.5	8.5	11	11	12	6	7	9.5	95.5
10	12	7	1	2	2	5	12	10	1	1	4	2	59
11	3	2.5	13	13	13	13	6	7	11	8	13	13	115.5
12	9	5	8.5	11	10.5	12	5	2	7	7	2	3.5	82.5
13	5.5	10	6	6.5	10.5	10	9	12	8	5	4	9.5	96

[a] Laboratory eliminated because of low bias.

Table 5. Ranked collaborator results for AAS method

Coll.	Sample 1	2	3	4	5	6	7	8	9	10	11	12	Total rank
1	8.5	9	12	9	7	5	5	6.5	11	12	11	9	105
2	3	3	3	3	10	13	2	2.5	2.5	2	4	10	58
3	2	2	5	2	1	3	1	1	4	4	2	3	30[a]
4	13	13	6	4.5	11	12	12.5	12.5	5	8.5	5	8	111
5	1	1	1	13	2	1	12.5	6.5	13	6	1	1	59
6	8.5	7.5	4	4.5	6	2	7	2.5	2.5	3	8	2	57.5
7	5	6	9	11	9	7	3	4	6	10	9	7	86
8	4	4	13	7	3.5	4	9	10	7	5	7	6	79.5
9	8.5	7.5	7	10	8	9	4	9	12	11	12	12	110
10	12	11	2	1	3.5	8	10.5	12.5	1	1	6	5	73.5
11	11	10	11	12	12	10	10.5	11	9	13	13	11	133.5[b]
12	6	5	8	6	5	6	7	6.5	8	8.5	3	4	73
13	8.5	12	10	8	13	11	7	6.5	10	7	10	13	116

[a] Laboratory eliminated because of low bias.
[b] Laboratory eliminated because of high bias.

Table 6. Within days (σ_W), between days ($\sigma_{D/L}$), between laboratories (σ_L), and total (σ_T) error estimates

Set No.	Samples	σ_{WD}		$\sigma_{D/L}$		σ_L		σ_T	
		FES	AAS	FES	AAS	FES	AAS	FES	AAS
I	1, 2 7, 8	0.0069	0.0070	0.0780	0.0111	0.0099	0.0018	0.0789	0.0143
II	3, 4 9, 10	0.0697	0.0828	0.0671	—[a]	0.0412	0.0758	0.1051	0.1122
III	5, 6 11, 12	0.1292	0.1565	0.1597	0.0406	—[a]	0.1181	0.2054	0.2002

[a] Value can not be estimated.

Table 7. Results of significance by *F*-test

Set No.	Samples	*F*-ratio							
		σ^2_{WD}	(df)[a]	$\sigma^2_{D/L}$	(df)	σ^2_L	(df)	σ^2_T	(df)
I	1, 2, 7, 8	1.03	(18, 20)	49.28[b]	(20, 18)	3.11[b]	(20, 18)	30.49[b]	(20, 18)
II	3, 4, 9, 10	1.41	(18, 18)			3.39[b]	(18, 18)	1.14	(18, 18)
III	5, 6, 11, 12	1.47	(18, 20)	15.50[b]	(20, 18)			1.05	(20, 18)

[a] df = degrees of freedom.
[b] Significant at the 95% level of confidence.

Table 8. Results of significance by *t*-test

Sample	Na, av. content (%)		Coeff. of var., %		Degrees of freedom	*t*-value
	FES	AAS	FES	AAS		
1	0.1213	0.0959	13.85	15.12	19	3.687[a]
2	0.1607	0.1294	7.65	14.21	19	4.470[a]
3	2.3658	2.2825	4.63	5.62	18	1.561
4	3.0234	2.9495	4.24	3.18	18	−0.997
5	7.8784	7.8779	1.59	2.15	19	0.007
6	8.6263	8.6497	2.73	2.54	19	−0.235
7	0.1174	0.1018	10.22	11.98	19	2.951[a]
8	0.1585	0.1321	7.95	7.42	19	5.312[a]
9	2.3189	2.3137	3.89	4.73	18	0.116
10	2.9567	2.8865	2.53	3.28	18	1.839
11	7.8833	7.8495	2.80	2.25	19	0.384
12	8.6196	8.5074	1.62	2.55	19	1.403

[a] Significant at the 95% level of confidence.

Three collaborators commented on the linearity of the working curve, which is better for the AAS method than for the FES method. One collaborator commented that a high potassium concentration enhances the sodium value obtained by the FES method for Samples 1, 2, 7, and 8. Another collaborator said that using a large sample size or smaller dilution would probably give better results for Samples 1, 2, 7 and 8.

Six of the 13 collaborators reported that they had to make a further dilution on Samples 3, 4, 9, and 10 to fit the calibration curve, because they were above 200 ppm Na. It can be assumed that the others also made this dilution unless they increased the calibration curve range or made an extrapolation.

Conclusions and Recommendations

The statistical analysis shows that the performance of the AAS method is better than that for the FES method in samples with a low sodium content and high potassium content, probably due to interferences caused by this element when the K/Na ratio increases. At K/Na molar ratios less than 4:1 (Sample 3), it appears that there is no influence on the sodium determination. In samples with high sodium content, there were no differences between the averages obtained by the 2 methods.

It is recommended that the AAS method be adopted official first action for determining sodium in fertilizers, extending the dilution for samples containing 4–20% to 1–20% Na to bring all solutions into an adequate range in the calibration curve. If this dilution is not carried out, samples with 2–4% Na will be above 200 ppm and those with 1–2% will be in a nonlinear section of the calibration curve, causing unreliable results. The calculation equations should also be changed to include samples with 1–4% Na.

It is also recommended that a further study be carried out on the influence of potassium in the determination of sodium by flame emission and means to eliminate it.

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Flame Photometric Determination of K_2O in Fertilizers: Collaborative Study

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A collaborative study of the flame photometric determination of K_2O in fertilizers has been conducted. The proposed method is intended to replace both the official manual flame method, 2.091, and the official automated method, 2.097. This is possible because the method is written in a general fashion in terms of instrument performance, rather than specifically for a particular instrument configuration. An example automated instrumental system in specific detail, which meets the general method requirements, is also included. Results for 20 samples from 11 laboratories were compared with results by the official STPB method, 2.102. Means and standard deviations of results are comparable between methods. Analysis of variance does not show any difference in methods. The method has been adopted official first action.

A collaborative study on the automated flame photometric determination of K_2O in fertilizers, reported in 1971 (1), resulted in the present official automated method, **2.097** (2). Because the method was written in rather specific terms for the most commonly used instrument at that time, new flame photometer models have made that method limited or obsolete.

Instead of again collaborating a modified method suited to a particular instrument model it was decided to write a method in general terms, allowing various instrument models and designs, but requiring specified performance checks on whatever instrument is used. The Associate Referee worked with the Committee on Automated Methods of the AOAC to develop a philosophy for appropriate performance criteria which might apply to this method and to automated methods in general.

Starting with the particular automated instrumentation currently in use in the Associate Referee's laboratory, included in this paper under the heading "Example Automated Instrument System," an attempt was made to keep the basic chemistry fixed but to generalize the instrument parameters as much as possible. The final performance criteria developed for the K_2O method are such that either an automated or manual flame instrument may be used. The type of automation could be either continuous flow or flow injection.

The method as sent to collaborators included 2 parts: a general section, specifying the parameters for the basic chemistry of the method and describing the various performance checks that must be carried out, and a second part consisting of detailed instructions for setting up and using the particular flame photometer and automated instrument system used in the referee's laboratory. This system is capable of meeting all the specified performance criteria. In the performance specifications section of this paper, the values that appear in parentheses indicate the performance capabilities of the example automated instrument system. Collaborators were free to choose this instrument system or another of their own design.

This method format has several advantages. First, it avoids specifying a particular instrument manufacturer within the context of an official method, or requiring that a particular instrument be purchased in order to perform the official method. This has been of concern to AOAC. Second, new developments in instrumentation can be quickly adapted without the long delays of new collaborative studies. As long as the chemistry is unchanged, all that is required is that the instrument's performance be suitably demonstrated. This should be of interest to instrument manufacturers. And finally, this method format provides a framework in which instrument manufacturers, referees, or other interested persons may make known various instrumental configurations which have been found to better meet all of the performance specifications required by the method. Instru-

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mental system improvement is thus encouraged rather than prohibited.

Twenty samples used in the collaborative study consisted of 10 blind duplicates arranged as 5 closely matched pairs. The samples were to be analyzed one time each by the official STPB method, **2.102** (2), by the flame photometric method using ammonium citrate extraction, and by the flame photometric method using ammonium oxalate extraction. Collaborators were asked to return performance check data and instrument system descriptions in addition to sample data.

K_2O in Fertilizers
Flame Photometric Method
(Manual or Automated)
Official First Action

(*Caution:* See **51.007**)

2.D06 *Method Parameters*

Any flame photometer, manual or automated, capable of detecting K, using Li as internal std, and meeting method performance characteristics described below, is satisfactory. Samples are extd with ammonium oxalate soln or ammonium citrate soln. Appropriate dilns of ext are mixed with $LiNO_3$ internal std soln and aspirated or pumped into flame photometer. La_2O_3 is added to $LiNO_3$ soln to eliminate the phosphate effect. Final soln to be introduced to flame should have the following composition: (a) concn of K_2O in range such that std curve response is linear over that range, (b) const amt of Li in range 5 to 40 ppm, (c) selected concn of La <1400 ppm, and (d) 0.2N HNO_3. Exact concn of $LiNO_3$ and La_2O_3 are optimized for particular instrumentation as described in performance specifications below. Ratio of K intensity at 768 nm to Li intensity at 761 nm is detd, and compared with similar ratios from std set of ≥6 stds, prepd from NBS or primary std KH_2PO_4. Stds are arranged in ascending order and evenly distributed thru chosen range.

2.D07 *Preparation of Sample*

(**a**) *Ammonium oxalate extraction.*—Weigh 1 g sample into 500 mL vol. flask, add 50 mL 4% $(NH_4)_2C_2O_4$ and 125 mL H_2O, boil 30 min, and cool. Dil. to vol. with H_2O, mix, and filter or let stand until clear.

(**b**) *Ammonium citrate extraction from direct available phosphorus extract.*—Prep. as in **2.050.** (If solns must be held overnight, add 3-4 drops of $CHCl_3$.)

2.D08 *Performance Specifications*

System performance criteria.—Detailed example of specific instrumental system capable of meeting specified performance criteria follows this performance section. It is necessary to verify that this or any other particular system meets all of the following performance criteria before samples are analyzed. Levels specified are to be considered min. acceptable levels. Various criteria are written for automated instrument, but should also apply to manual instrument systems.

(**a**) *$LiNO_3$ concentration level.*—Amt of $LiNO_3$ in final soln aspirated into flame is adjusted partly for convenience of instrument parameters, but should be such that Li and K channels give roughly equal responses. This can be detd either by displaying each channel's output sep., or by displaying ratio of K to Li response and then interchanging Li and K filters and displaying ratio again.

Using either procedure, sample midrange K^+ stds under analysis conditions while varying concn of Li^+ until acceptable concn of Li^+ is found.

(**b**) *Noise.*—Adjust detector output to 90% full scale with high std sampled continuously. Noise must be <2% full scale peak to peak. Note that some instruments, flow injection analysis systems, for example, are not designed to pump samples continuously. In this case, substitute repeated sampling for continuous sampling, and consider noise to be difference between adjacent peak maxima. (Optimum performance on example instrument system described in this method is ca ½% peak to peak. To reduce noise on example system, stabilize flame, stabilize pumping rate, stabilize back-pressure, change pump tubes, clean manifold, and/or rework manifold to ensure adequate mixing. To det. min. noise limit of instrument, collect system waste soln, connect short length of tubing directly to photometer aspirator, and aspirate waste soln directly into flame.)

(**c**) *Carryover.*—Adjust detector output to give ca 10% and 90% full scale response for low and high stds, resp. Sample 3 high stds followed by 5 low stds on system under analysis conditions. Carryover, defined as difference between first low std and mean of other low stds, may not be >1% full scale. (Optimum performance on example system is negligible carryover. To reduce carryover on example system, clean manifold

and aspirator, check manifold connections for dead space, redesign manifold shortening hydraulic system wherever possible, decrease sampling rate, and/or reduce std range.)

(d) *Drift.*—Adjust instrument to give detector response ca 50% full scale with middle std sampled continuously. Sample middle std continuously for the time it would take to analyze 30 samples. For instruments not designed to sample continuously, draw smooth line thru 30 middle std peaks. Drift may not exceed 1% full scale per any 10 sample segment. (Optimum performance on example system is zero drift. To reduce drift on example system, stabilize room and soln temps, adjust manifold to maintain const back-pressure, and/or stabilize flame.) As long as drift does not exceed 1% per 10 peak level, routine data may be further improved by inserting a middle std periodically between groups of samples. This allows mathematical peak ht correction, assuming linear drift.

(e) *Precision.*—With instrument calibrated for 10% and 90% full scale for low and high stds, resp., sample 30 middle stds under analysis conditions. Range of instrument response may not vary >2% full scale. (Optimum performance on example system is 0.7% full scale. To improve precision on example system, reduce noise, check sampler timing, and/or decrease sampling rate.)

(f) *Std curve.*—Std curve consists of ≥6 different stds, evenly distributed thru std concn range. Prep. solns from NBS or primary std KH_2PO_4, dried 2 h at 105°. Include factor for actual purity of std material in calcns of std concn.

With instrument calibrated for ca 10% and 90% response for low and high stds, resp., run stds in order of ascending concn under analysis conditions. Response should be linear. Mathematically perform first degree least squares fit to std curve data. Alternatively, use calculator capable of least squares fits. First order least squares fit may be performed as follows: Assume that points to be fitted are $(X_1, Y_1), (X_2, Y_2), \cdots (X_n, Y_n)$. Calc. means by:

$$\overline{X} = \frac{1}{n}\Sigma X_i \quad \overline{Y} = \frac{1}{n}\Sigma Y_i$$

Slope of least square fitted line is given by:

$$b_1 = \frac{\Sigma(X_i - \overline{X})(Y_i - \overline{Y})}{\Sigma(X_i - \overline{X})^2} = \frac{(\Sigma X_i Y_i) - n\overline{X}\overline{Y}}{(\Sigma X_i^2) - 1/n(\Sigma X_i)^2}$$

Intercept for line is given by:

$$b_0 = \overline{Y} - b_1\overline{X}$$

Equation of resulting line is:

$$Y = b_0 + b_1 X$$

Using derived equation and individual std responses, calc. concn for each std. Compare calcd and known concns for each std. Calcd value may not differ from known value by >±2% in any one instance. Also, av. of absolute values of those % differences may not be >1%. (Optimum performance on example system is 0.75% and 0.37%, resp. To improve std curve fit, optimize parameters (b) thru (e) above and/or reduce std range.)

(g) *Phosphate effect.*—For example system, amt of La_2O_3 in $LiNO_3$ reagent is sufficient to eliminate phosphate effect (depression of instrument response to K by phosphate ion). If other than example automated system is used, elimination of phosphate effect must be verified. Using KNO_3, prep. 200 mL soln of K_2O with concn equal to twice that of highest std. Pipet 50 mL of that soln into each of two 100 mL vol. flasks. Dil. one to vol. and mix. Add sufficient $NH_4H_2PO_4$ soln to the other flask such that concn of P_2O_5 will be as high as highest concn of P_2O_5 anticipated in any sample ext. Dil. to vol. and mix. Sample 10 portions of each soln, alternating, under analysis conditions. Average 10 responses for each soln. Av. responses of the 2 solns must not differ from each other by >1%. Select min. amt of La_2O_3 which will eliminate phosphate effect. (Optimum performance of example system is <0.5%. To improve performance, adjust amount of La_2O_3.)

(h) *Overall performance of system.*—Performance characteristics mentioned above are worst case examples. A system functioning marginally in many categories would probably fail the following overall performance check.

Verify overall performance as follows: Ext and analyze once each 20 different Magruder samples, or other similar performance check samples previously detd by interlaboratory study. Also ext and analyze 5 independent 1 g portions of NBS or primary std KH_2PO_4. Randomize Magruder and KH_2PO_4 sample order. Calc. % K_2O. Av. bias of Magruder results, Σ (Magruder grand av. − calcd % K_2O)/20, must be <±0.1. Av. of absolute value of differences must be <0.4. (Optimum values on example system are ca ±0.02 and ±0.2, resp.)

For 5 analyses of KH_2PO_4, difference between mean of calcd % K_2O and known % K_2O must not be >±0.2, and std deviation must not be >0.25. (Optimum values for example system are ±0.1 and 0.15, resp.)

(i) *Ongoing performance checks.*—(*1*) Conduct daily performance check by analyzing same performance check sample at least once in every 60 regular samples, and at least once in each run. (*2*) Repeat (**h**) above at least twice per year, and whenever system has not been used for prolonged periods.

Example Automated Instrument System

2.D09 *Apparatus*

Automatic analyzer.—AutoAnalyzer with following modules (available from Technicon Instruments Corp.): sampler II or IV, pump III, flame photometer IV, and recorder. Computer or calculator capable of least square fits is desirable.

2.D10 *Reagents*

(**a**) *Ammonium oxalate soln.*—Dissolve 40 g $(NH_4)_2C_2O_4$ in 1 L H_2O.

(**b**) *Ammonium citrate soln.*—Should have sp. gr. of 1.09 at 20° and pH of 7.0 as detd potentiometrically.

Dissolve 370 g cryst. citric acid in 1.5 L H_2O and nearly neutze by adding 345 mL NH_4OH (28–29% NH_3). If concn of NH_3 is <28%, add correspondingly larger vol. and dissolve citric acid in correspondingly smaller vol. H_2O. Cool, and check pH. Adjust with NH_4OH (1+7) or citric acid soln to pH 7. Dil. soln, if necessary, to sp. gr. of 1.09 at 20°. (Vol. will be ca 2 L.) Keep in tightly stoppered bottles and check pH from time to time. If pH has changed from 7.0, readjust.

(**c**) *Lithium nitrate soln.*—Dissolve 1.642 g La_2O_3 in 30 mL HNO_3, add 0.9935 g dried (2 h at 105°) $LiNO_3$ and 1 mL Flaminox 1% soln (Fisher Scientific Co.), and dil. to 1 L with H_2O.

(**d**) *Sampler wash and dilution water soln.*—Dil. 1 mL Flaminox 1% soln to 1 L with H_2O.

(e) *Potassium std solns.*—(*1*) *Stock std soln.*—1 mg K_2O/mL. Dissolve 2.889 g dried (2 h at 105°) KH_2PO_4 (NBS SRM 200) in H_2O, and dil. to 1 L. (*2*) *Working std solns.*—10, 20, 30, 40, 50, and 55 μg K_2O/mL. Accurately measure by buret 10, 20, and 30 mL stock std soln into 1 L vol. flasks, and 20, 25, and 27.5 mL into 500 mL vol. flasks. Add 0.2 g $(NH_4)_2C_2O_4$ per 500 mL final vol. if samples are prepd by ammonium oxalate extn, or add 12 mL ammonium citrate soln per 500 mL final vol. if samples are prepd by ammonium citrate extn. Dil. to vol. with H_2O and mix. (Add 3 mL $CHCl_3$ to preserve citrate std solns for long periods.)

2.D11 *Analytical System*

Assemble manifold as in Fig. **2:D1**. Use 1.6–2.0 mm id glass transmission tubing for all reagent flow upstream from D1 fitting. Use clear std pump tubes for air and soln stream flow.

Air and H_2O are combined thru injection fitting (116-0492-01). Hard thin-wall polyethylene tubing (ca 0.30 in. id) connects air bar tubing to injection fitting. Sample is introduced immediately downstream thru second injection fitting (194-G012-01), designed to eliminate double peaks in recorder output. Mixing of sample and H_2O occurs in double 10-turn coil with insert (157-B089). $LiNO_3$ reagent is introduced thru insert. Another 10-turn coil (157-0251) further mixes solns.

Portion of soln is aspirated to flame photometer thru A4 fitting (116-0200-04). Hard, thin-wall polyethylene tubing (ca 0.045 in. id) connected to photometer is inserted and glued to tee arm of A4 fitting. Remaining unaspirated soln is drawn thru double 10-turn mixing coil (157-0248-01) and thru D1 fitting (116-0203-01). Large diam. branch of D1 fitting leads to pump and waste. Small diam. branch of D1 fitting is connected to 6 ft (1.83 m) of Tygon tubing (0.030 in. id) to waste. D1 fitting is oriented with small diam. branch low, so that only soln, and no air, enters 0.030 in. tubing. This establishes const back pressure and therefore stable aspiration conditions at flame photometer.

2.D12 *Startup and Shutdown Procedures*

Start system and place reagent lines in proper solns. Let equilibrate 30 min before beginning calibration. Adjust flame photometer as follows: (*1*) damping control to damp 3 position; (*2*) flame ht of main cone ca 4 cm; (*3*) atomizer adjust control set to give atomization rate of ca 1.3–1.4 mL/min. Rate of atomization is detd by subtracting rate of flow to waste from rate of flow upstream from (disconnected) A4 fitting. Use 0.3 and 0.6 neut. density filters for Li and K detectors, resp.

Initially it may be necessary to manually fill system downstream from A4 fitting with H_2O, making certain that 6 ft of 0.030 tubing is filled. System is shut down after pumping H_2O thru reagent lines ≥15 min.

2.D13 *Checkout and Calibration*

After equilibration, pump 10 μg K_2O/mL std thru system and adjust baseline control of photometer to read 10% full scale. Pump 55 μg

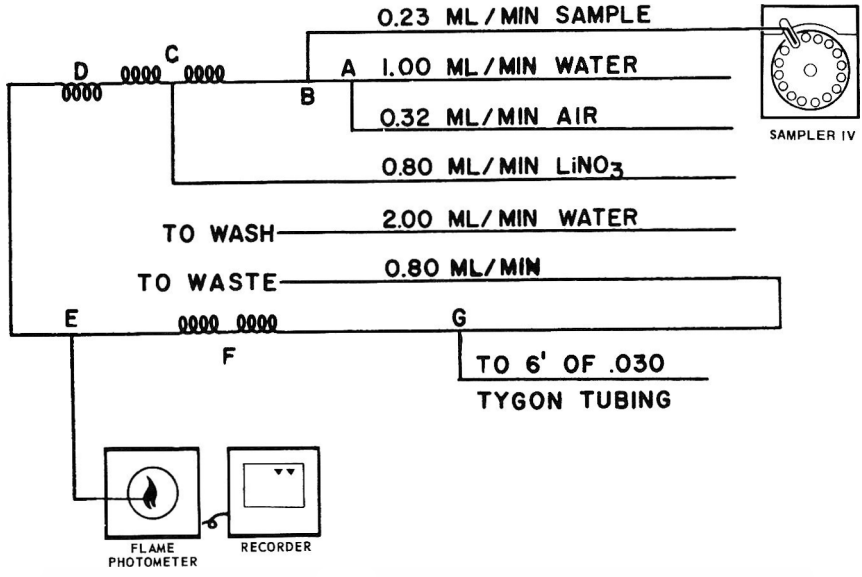

Figure 2:D1. Manifold for K_2O in fertilizers. A, injection fitting 116-0492-01; B, injection fitting 194-G012-01; C, double 10-turn coil with insert 157-B089; D, 10-turn coil 157-0251; E, A4 fitting 116-0200-04; F, double 10-turn coil 157-0248-01; G, D1 fitting 116-0203-01.

K_2O/mL std thru system and adjust std calibration control to read 90% full scale. If noisy conditions exist, check for aspiration of air at A4 fitting, or check for air entering lower arm of D1 fitting. If drift exists, check room and solns for temp. stability. Std curves should be virtually linear.

2.D14 *Determination*

Pipet aliquots of sample solns, Table **2:D1,** into 250 mL vol. flask, dil. to vol. with H_2O, and mix 15 times. For 10 mL aliquots of citrate extns, add 4 mL ammonium citrate soln to aliquots before dilg to vol. Run samples in groups of 10. Place 10 thru 55 μg K_2O/mL stds in order in sampler, preceded by extra 10 μg/mL std. Place 30 μg/mL std after every 10th sample, to be used for drift correction. End series with two 30 μg/mL stds. Sample at rate of 40/h, 2:1 sample-to-wash ratio.

Table 2:D1. K_2O aliquots

% K_2O expected	Aliquot, mL
<2	250 (no diln)
2–6.49	100
6.50–19.99	30
≥20	10

2.D15 *Calculations*

Correct sample peak hts for drift. Correct peak hts of first 10 samples as follows:

$$H_c = H - [(D_1 - D_0)/14][L + 3]$$

where H_c = corrected peak ht; H = uncorrected peak ht; D_1 = ht of first drift correction std; D_0 = ht of 30 μg std in initial std sequence; and L = position No. of sample peak to be corrected. Correct subsequent sample peak hts as follows:

$$H_c = H - [D_x - D_0] - [(D_y - D_x)/11][P]$$

where D_x = ht of drift std preceding sample to be corrected; D_y = ht of drift std following sample to be corrected; and P = position No. of sample within group of 10.

Calc. least squares fitted curve of emission against K_2O concn. Calc. μg K_2O/mL of corrected peak hts from equation:

$$\% K_2O = (\mu g\ K_2O/mL \times 12.5)/(\text{aliquot} \times \text{g sample})$$

Results and Discussion

Table 1 lists the collaborative samples, identifying the blind duplicates. As much as possible, closely matched pairs were selected from the same source to have similar matrices.

Collaborative results were received from 11

Table 1. Collaborative samples

Blind duplicates, Sample	Closely matched pairs	Grade	Source
1, 9	Pair 1	15-40-5	Company A
6, 13		15-40-5	Company A
2, 15	Pair 2	reagent grade KCl	Fisher Sci. Co.
4, 18		0-0-62	composite mixt.
3, 11	Pair 3	primary std KH_2PO_4	Fisher Sci. Co.
5, 20		4-12-36	Company B
7, 17	Pair 4	6-24-24	Company B
10, 19		6-24-24	Company B
8, 16	Pair 5	12-12-12	Company B
12, 14		12-12-12	Company B

laboratories. Table 2 presents the results for the STPB method, Table 3 for the flame photometric method with ammonium citrate extraction, and Table 4 for the flame photometric method with ammonium oxalate extraction.

Collaborator 7 used an Instrumentation Laboratories No. 951 manual flame instrument without lithium internal standard capability. The overall performance check was not within specifications. Collaborator 10 used a Varian Model AA-5 instrument operating in the absorption mode. He found that he could not get linear standard response in the emission mode. Also, in the ammonium citrate extraction, he omitted the water wash step. Collaborator 11 submitted results only for the ammonium oxalate extraction, and provided no performance check data and no indication of type of instrument used. Collaborator 2 used a Perkin-Elmer 503 instrument in the emission mode. Collaborator 8 used an Instrumentation Laboratories No. 353 manual AA/AE spectrometer in the emission mode.

Collaborators 1 and 6 used the example automated method provided as an option with the collaborative materials. Collaborators 4 and 9 used the example method with minor modification, including Brij® (Technicon) as the wetting agent instead of Flaminox® (Fisher). Collaborator 5 used a Technicon Flame III instrument essentially as outlined in the present automated official method.

Collaborator 3 used a Technicon Flame IV instrument with a modified manifold design and wider range standards to eliminate aliquoting the original sample extracts. This manifold design resulted in a final concentration of about 17 ppm K_2O for aspiration into the flame. The maximum concentration proposed in the collaborative method was 6.25 ppm K_2O. Other than this, Collaborator 3 had acceptable performance levels and results.

The Associate Referee's laboratory subsequently tested a similarly designed manifold and also found it to have acceptable performance checks, with linear standard curves. It would appear that the 6.25 ppm upper limit on K_2O concentration is too restrictive.

Two-sample X-Y charts (3) were prepared for each closely matched pair for each of the 3

Table 2. Collaborative results (% K_2O), STPB method

	Collaborator								
Sample	1	2	3	4	5	6	7	8	9
1	5.94	6.00	6.05	5.80	5.91	6.33	6.05	6.13	6.16
2	62.85	63.99	62.82	62.72	62.38	63.73	63.08	63.56	63.24
3	34.33	35.09	34.32	34.56	34.80	34.78	34.79	34.79	34.61
4	61.57	62.03	61.83	61.68	62.11	62.77	61.69	62.47	62.13
5	34.65	34.72	34.53	34.54	34.99	34.88	34.79	34.87	34.72
6	6.02	6.11	5.96	5.71	5.97	6.17	5.94	6.06	5.92
7	24.30	24.80	24.44	24.40	24.54	24.61	24.63	24.56	24.64
8	12.83	13.08	12.87	12.79	12.95	13.21	13.19	13.01	12.91
9	5.94	6.13	6.11	5.76	6.05	6.24	6.04	6.28	6.08
10	24.57	24.93	24.44	24.34	24.07	24.63	24.50	24.59	24.72
11	34.37	35.41	34.64	34.48	34.43	34.61	34.57	34.71	34.64
12	14.84	15.01	15.12	14.68	14.95	15.14	14.71	14.97	14.84
13	5.84	5.97	5.99	5.77	5.95	6.15	5.99	6.15	5.94
14	14.72	14.87	15.11	14.68	14.93	15.05	14.83	14.97	14.82
15	62.35	63.16	63.04	63.08	62.76	63.62	62.85	63.53	63.21
16	12.83	13.02	12.85	12.68	12.83	13.10	12.73	13.04	12.92
17	24.23	24.94	24.42	24.36	24.23	24.48	24.86	24.70	24.53
18	61.42	62.65	61.90	61.76	61.05	62.23	61.99	62.53	62.03
19	24.15	24.97	24.52	24.26	24.65	24.55	24.59	24.80	24.61
20	34.55	35.69	34.64	34.40	34.56	34.65	34.76	34.68	34.65

Table 3. Collaborative results (% K_2O), flame photometry, ammonium citrate extraction

	Collaborator									
Sample	1	2	3	4	5	6	7	8	9	10
1	6.10		6.08	6.04	6.39	6.03	5.83	6.09	5.98	6.00
2	64.32		62.73	62.84	61.79	63.46	61.85	63.90	63.02	64.09
3	35.21		34.62	35.37	35.17	34.47	33.56	34.83	34.74	Lost
4	62.90		62.08	61.65	61.37	62.42	60.69	62.67	61.91	64.03
5	35.02		34.62	35.02	35.20	34.85	34.35	34.79	34.59	35.00
6	5.95		6.08	6.00	6.18	5.93	5.66	6.01	5.94	6.00
7	24.34		24.47	25.02	24.29	24.50	23.99	24.67	24.38	24.15
8	12.97		12.92	12.88	13.22	12.69	13.44	12.90	12.71	12.97
9	6.15		6.08	6.12	6.17	6.01	5.77	6.01	6.05	6.02
10	24.62		24.63	24.67	24.57	25.00	23.39	24.56	24.30	24.00
11	35.12		34.95	34.32	34.63	34.47	33.48	34.64	34.42	34.97
12	15.02		14.87	14.84	14.69	14.95	14.51	14.61	14.78	15.01
13	6.06		5.92	5.96	6.14	5.93	6.16	5.94	5.91	6.01
14	14.97		14.87	14.63	14.69	14.72	14.88	14.94	14.84	15.02
15	64.27		62.73	62.63	61.87	63.74	63.32	63.30	63.26	63.12
16	12.95		12.91	12.86	13.71	12.85	13.03	12.83	12.93	13.03
17	24.44		24.80	24.95	24.49	24.55	24.95	24.55	24.39	24.00
18	63.11		62.08	61.30	61.32	62.74	61.82	62.92	61.99	62.14
19	24.28		24.80	24.67	24.60	24.46	24.43	24.77	24.60	24.05
20	35.16		35.27	35.02	34.74	34.74	33.52	35.49	34.54	35.02

methods. Since Collaborator 7 did not use an internal standard instrument, Collaborator 10 detected in the absorption rather than emission mode, and Collaborator 11 did not include any performance check information or instrument description with the data, and since all 3 had a number of apparently extreme values on the X-Y charts, the flame photometric data for these 3 collaborators were excluded from further statistical analysis. All other data were kept.

For each concentration level and for each of the 3 methods, S_r, a measure of intralaboratory standard deviation, and S_d, a measure of overall interlaboratory standard deviation, were calculated according to Youden (4). The values, which are required for inclusion with official methods, are listed in Table 5. Since the collaborative scheme involved blind duplicates of closely matched pairs, each S_r and S_d is an average of 2 values. Table 5 also includes the num-

Table 4. Collaborative results (% K_2O), flame photometry, ammonium oxalate extraction

	Collaborator										
Sample	1	2	3	4	5	6	7	8	9	10	11
1	6.03	6.08	6.10	6.09	6.08	6.05	6.06	5.97	6.00	6.51	5.91
2	63.17	63.88	62.61	63.53	62.46	63.96	60.89	63.06	62.16	64.31	63.30
3	34.70	35.00	34.69	35.30	36.12	34.94	34.09	34.48	34.55	37.06	34.60
4	62.11	62.10	61.37	62.59	61.43	62.65	59.17	62.44	61.36	64.10	62.00
5	35.25	34.81	34.69	35.50	35.75	35.14	33.93	34.68	34.51	36.92	34.82
6	6.01	6.10	6.10	6.04	5.92	5.98	5.86	5.90	5.89	6.50	5.64
7	24.47	24.65	24.59	24.99	24.60	24.87	24.18	24.64	24.34	25.17	24.49
8	13.00	12.90	13.03	13.05	12.99	12.97	11.95	13.01	12.74	14.06	12.57
9	6.06	6.16	6.10	6.23	5.98	6.04	6.00	5.97	5.93	6.51	5.74
10	24.58	25.02	24.28	24.59	25.80	25.16	23.34	24.18	24.14	24.48	24.60
11	34.64	35.55	34.69	34.70	35.06	34.96	33.76	34.24	34.40	36.21	34.69
12	14.88	15.10	14.88	15.00	14.54	14.90	14.65	14.48	14.68	15.51	14.83
13	6.01	6.00	6.10	6.03	5.89	5.93	5.80	5.78	5.99	6.51	5.60
14	15.04	14.98	14.73	14.91	15.05	14.78	14.19	14.49	14.71	16.00	14.71
15	64.35	63.12	63.22	63.26	62.98	63.74	63.61	63.17	62.80	64.08	62.30
16	13.09	13.18	12.87	13.30	13.13	12.93	12.22	12.71	12.81	13.52	12.59
17	24.58	25.07	24.43	24.99	24.87	24.72	23.67	24.37	24.22	25.07	23.32
18	62.53	62.67	61.99	63.46	61.37	62.55	58.67	61.89	61.54	63.09	61.50
19	25.01	25.05	24.74	25.13	25.75	24.89	25.36	24.51	24.21	24.91	23.57
20	35.03	35.41	35.00	35.37	34.92	34.98	33.86	34.08	34.51	36.01	33.13

Table 5. Means (% K_2O), S_r, S_d, and CV values by concentration level

Statistic	STPB method 18 Pairs	Ammonium citrate method 14 Pairs	Ammonium oxalate method 16 Pairs
Mean	6.017	6.045	6.017
S_r	0.070	0.043	0.056
S_d	0.144	0.095	0.085
CV, %	2.39	1.57	1.41
Mean	13.919	13.865	13.902
S_r	0.113	0.159	0.121
S_d	0.150	0.142	0.179
CV, %	1.08	1.02	1.29
Mean	24.543	24.581	24.733
S_r	0.139	0.176	0.299
S_d	0.226	0.212	0.322
CV, %	0.92	0.86	1.30
Mean	34.700	34.858	34.895
S_r	0.112	0.191	0.251
S_d	0.271	0.300	0.480
CV, %	0.78	0.86	1.38
Mean	62.550	62.654	62.673
S_r	0.279	0.257	0.3556
S_d	0.457	0.735	0.597
CV, %	0.73	1.17	0.95

Table 6. Standard deviations of results by method at various concentration levels

Sample	Approx. concn, % K_2O	Ammonium oxalate SD	Ammonium citrate SD	STPB SD
1, 9	6	0.077	0.101	0.151
6, 13	6	0.090	0.089	0.125
8, 16	12	0.156	0.145	0.151
12, 14	12	0.201	0.132	0.150
7, 17	24	0.253	0.223	0.204
10, 19	24	0.511	0.187	0.239
3, 11	35	0.471	0.332	0.270
5, 20	35	0.429	0.382	0.278
4, 18	60	0.613	0.628	0.442
2, 15	60	0.576	0.779	0.450

ber of pairs used to determine the standard deviations, the means, and the coefficients of variation.

In general, the S_r and S_d values are either comparable to or lower than the corresponding values for the same method and concentration range from the previous study (1). A case might be made that the standard deviations at the 60% level are slightly higher than in the previous study. But this condition applies across all 3 methods.

A noteworthy trend appears if standard deviations of results by method at various guarantee levels are examined as in Table 6. Comparing the STPB and ammonium citrate method standard deviations, the STPB method has more overall precision at the higher guarantee levels while the ammonium citrate method has more precision at the lower guarantee levels. At the midrange levels, the 2 are roughly equivalent. In general, the ammonium oxalate standard deviations fit this same trend, except several midrange deviations appear abnormally high.

This situation is no doubt due to the basic design of the methods. If desired, the flame photometric methods could be made more precise at the higher guarantee levels by adjusting the standard curve range and aliquoting scheme. This becomes a quite simple matter to do when the method is written in terms of performance, as the flame photometric method is. An individual laboratory could and should tailor the method to its specific wants and needs.

Two-way factorial analyses of variance, method by laboratory segregated by sample, were performed. For Sample 5, the 4-12-36 sample, Table 7, there was a significant method effect. The average result across all laboratories for the ammonium oxalate method was 34.96%, for the ammonium citrate 34.93%, and for the STPB 34.66%. It appears that the STPB method presented difficulty with this particular sample. The product consisted of ammonia, phosphoric acid, triple super, sulfuric acid, and potassium chloride. No reason for this discrepancy between methods could be deduced from the sample components.

A 2-way factorial analysis of variance, method by sample across all laboratories, was performed (Table 8). Since standard deviations had a linear

Table 7. Analysis of variance, method by laboratory, Sample 5

Source	Sum of squares	DF	Mean square	F	Significance of F (a)
Method	0.736	2	0.368	5.696	0.011
Laboratory	0.816	6	0.136	2.105	0.096
Interaction, method X lab.	1.734	12	0.144	2.235	0.051
Residual	1.357	21	0.065	—	—

Table 8. Analysis of variance, method by sample across all laboratories

Source	Sum of squares	DF	Mean square	*F*	Significance of *F* (a)
Method	0.001	2	0.000	2.635	0.073
Sample	0.406	9	0.045	278.163	0.001
Interaction, method × sample	0.003	18	0.000	1.031	0.423
Residual	0.063	390	0.000	—	—

relation with concentration level, results were scaled to unity so that standard deviations were uniform across all sample levels. The *F*-value for method was not significant.

Conclusions and Recommendation

The flame photometric methods give good agreement with the STPB method, with the exception of Sample 5 and the STPB method. Values for S_r and S_d for all 3 methods are generally equivalent or better than corresponding values in the 1971 collaborative study which established the automated method as official. A possible exception occurs at the 60% level, where the STPB method as well as the flame photometric methods had somewhat larger standard deviations than in the 1971 collaborative study. Whether or not this is significant, the flame photometric methods may be easily adjusted for higher precision at the 60% guarantee level within the framework of the proposed method if desired.

The specified limit of 6.25 ppm K_2O as the maximum concentration of potassium to be aspirated into the flame was arbitrarily set at too small a value. Subsequent work has shown that instrument configurations can be devised meeting all performance criteria while aspirating about 18 ppm K_2O.

It is recommended that the proposed flame photometric method for the determination of K_2O in fertilizer be adopted official first action; and that the 6.25 ppm maximum concentration restriction for the standard curve be removed, and instead it be specified that the K_2O standard concentrations be such that the standard curve response is linear.

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FRUIT AND FRUIT PRODUCTS

Composition of Apple Juice

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Thirty-one samples from 8 geographic growing regions of the United States and 15 varieties common to these areas were converted to apple juice and analyzed for their attributes over the 3 year period 1979, 1980, and 1981. The total of 93 samples were analyzed for ash, brix, pH, proline, specific gravity, total acid, sorbitol, sucrose, fructose, and glucose. The elements cadmium, calcium, iron, lead, phosphorus, potassium, sodium, and zinc were also determined. These data are presented to serve as a data base for the detection of fraudulent or adulterated apple juice.

It has long been recognized that the chemical composition of apple juice varies depending on the cultivar, growing region, climate, maturity, and cultural practices. Only limited information is available, however, that shows the effect of these different variables on the composition of apple juice produced in the United States (1)

During the past 10 years, the consumption of apple juice has increased markedly, until it is now the leading single strength fruit juice consumed in this country. It is a concern of the apple juice processing industry that the popularity of their product could cause problems of adulteration. This project was initiated, therefore, to obtain a data base regarding juice composition that would be useful in combatting this type of fraud.

Experimental

Source of the apples.—The geographic source and varieties of apples used in this study are listed in Table 1. Apples were representative of the material normally entering an apple processing plant. Each of these varieties from the location designated was shipped every year between 1979 and 1981 to the New York State Agricultural Experiment Station at Geneva, NY.

Processing the apples. When received, apples were taken to the pilot plant of the Department of Food Science and Technology and the unsound fruit were removed. Sound fruit were then ground and the juice was extracted using a continuous screw press. The resulting juice was treated with pectic enzyme and filtered, using a plate and frame filter precoated with diatomaceous earth. The juices were bottled after being passed through a heat exchanger, which heated the product to 88°C. These pilot plant operations used conditions which closely approximated commercial conditions.

Samples were stored at 6°C until analyzed, which was done within a month after processing.

Ash, brix, and specific gravity analysis.—These constituents were analyzed as described by AOAC (2). Ash analysis was performed using method **31.012.** Soluble solids of the juice was determined by the method contained in **22.024** and **13.011,** with no correction being made for insoluble substances. Specific gravity was calculated using the pycnometer technique, **9.009-9.011.**

pH and total acid analysis.—A 10 mL sample of the juice was placed in a 250 mL beaker and pH was measured using a combination glass electrode and an Orion Research Digital Ionalyzer, Model 501, equipped with an automatic temperature probe. Following the pH measurement, the sample was agitated with a magnetic stirrer and titrated with 0.1N NaOH to an end point of pH 8.1. The total acid was calculated as percent malic acid.

Proline analysis.—Proline was determined by the method described by Amerine and Ough (3).

Sucrose, glucose, fructose, and sorbitol determination.—These components were determined by high pressure liquid chromatography (HPLC) using a Model 7000 Micromertics HPLC instrument equipped with a Waters Model R-401 differential refractive index detector. The detector and a strip chart recorder (Fisher Recordall 5000) were connected in parallel to a dedicated computer (Type 3353, Hewlett-Packard Corp.) suitable for the acquisition of laboratory data. The sensitivity of the detector was set at 64X. The combination of 64X and 1 mV full scale on the recorder was used to interface the detector with the data acquisition system. The recorder

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Table 1. Geographic source and apple varieties used in study of apple juice authenticity

State	Varieties
New York	Baldwin, Cortland, Idared, McIntosh, Rhode Island Greening, Twenty Ounce
California	Golden Delicious, Gravenstein, Jonathan, Rome Beauty, Yellow Newton
Washington	Granny Smith, Jonathan, Red Delicious, Winesap, Yellow Newton
Michigan	Cortland, Jonathan, McIntosh, Red Delicious
Pennsylvania	Golden Delicious, Jonathan, Red Delicious, Stayman
Massachusetts	Cortland, McIntosh, Red Delicious
North Carolina	Golden Delicious, Red Delicious, Rome Beauty
Virginia	York Imperial

was operated at a chart speed of 0.5 cm/min. A carbohydrate analysis column, 300 mm × 7.8 mm containing Aminex HPX-87C, 9 μm particle size (Bio-Rad Laboratories, Richmond, CA), was maintained at 86°C. Detector temperature was maintained at 20°C, using a Model FJ Haake constant temperature water circulator. Deionized water was used as the mobile phase. The reservoir temperature was maintained at 65°C.

A standard solution containing 5.0 μg/μL each of the components was prepared. A 10.0 μL sample was injected, and the response to each individual component was calibrated using the external standard method.

Apple juice samples were prepared by passing 5.0 mL juice through 4 mL of an anion exchange resin (AG1-X8, hydroxide form, Bio-Rad Laboratories) contained in a disposable chromatographic tube with a solvent reservoir (Cat. No. 5-8101, Supelco, Inc., Bellefonte, PA). Deionized water was passed through the column and 50 mL eluate was collected in a volumetric flask.

Twenty μL collected sample was injected into the HPLC instrument. The concentration of the components in the original apple juice was calculated as follows:

g Sugar or sorbitol/100 mL apple juice = μg determined × dilution factor/10 × μL sample injected

Calculation of fructose:glucose ratio before and after inversion of sucrose.—The fructose:glucose ratio is calculated by using the original analytical data for the components in the apple juice.

A theoretical level of glucose and fructose after inversion was calculated based on the stoichiometric relationship of the products, which are added to the original concentrations of the glucose and fructose. The calculation of the ratio after inversion is determined as follows:

Fructose:glucose (after inversion) = [(g fructose/100 mL) + 0.526(g sucrose/100 mL)]/[(g glucose/100 mL) + 0.526 (g sucrose/100 mL)]

Cation determination.—The cations calcium, iron, potassium, sodium, and zinc were determined using an atomic absorption spectrophotometer, Perkin-Elmer Model 305B, equipped with burner control. The spectrophotometer, when operated under the conditions outlined in Table 2, gave optimum recorder response and a peak duration of approximately 5 s for the standard solution.

Phosphorus determination.—Juice samples were subjected to wet ashing using perchloric and concentrated nitric acids to ensure the conversion to PO_4 ion before the colorimetric measurement by the molybdovanadate procedure of AOAC, **22.042-22.045** (2).

Cadmium and lead determination.—Cadmium and lead were determined by differential pulse anodic stripping voltammetry, using a Princeton Applied Research Corp. Model 174A instrument.

Table 2. Operating parameters for atomic absorption spectrophotometric determination of cations

Element	Ca	Fe	K	Na	Zn
Flow setting—fuel (mL/min)	25	25	30	30	25
—air (mL/min)	45	40	50	50	40
Vertical burner height	5.5	5.5	5.5	5.5	5.5
Source	10	30	12	8	15
Wavelength—(nm)	211	248	383	294	214
—(range)	vis	UV	vis	vis	UV
Slit	4	3	4	4	4
Recorder exp.	×3 or ×10	×10	×3	×3	×3
Dampening degree	Int 2 or 10	Int 2	Int 2	Int 2	Int 2
Upper limit (μg/mL)	5	5	2	1	1
Recorder range (mv)	5 or 20	5 or 10	5	10	5
Chart speed (mm/min)	20	20	20	20	20

The samples were prepared and measured using the method of Gajan and Larry (4).

Results and Discussion

The apple juices prepared from the apples received were analyzed for ash, brix, pH, proline, specific gravity, total acid, sorbitol, sucrose, fructose, and glucose as well as the elements cadmium, calcium, iron, lead, phosphorus, potassium, sodium, and zinc. The variations found on analysis of the juices for various components are listed in Table 3. In Table 4, the composite data have been calculated for the 3 years. The data do not show marked differences in the range of constituents between the years of the analysis. For all practical purposes, the mean, standard deviation, coefficient of variation, minimum and maximum for a single attribute are the same for all 3 years.

During the course of the study on single

Table 3. Mean, standard deviation, coefficient of variation, minimum, and maximum of authentic apple juice prepared over 3-year period (1979–1981)

Attribute	Mean	SD	CV, %	Min.	Max.
		1979			
Ash (%)	0.201	0.0454	22.6	0.13	0.30
Brix	12.60	1.85	14.7	10.1	16.9
pH	3.66	0.581	15.9	3.31	4.24
Proline (ppm)	5.97	2.38	39.9	2.88	12.65
Specific gravity	1.0473	0.0070	0.67	1.0372	1.0690
Total acid (% as malic)	0.406	0.172	42.4	0.15	0.79
Sorbitol (g/100 mL)	0.565	0.261	46.2	0.16	1.20
Sucrose (g/100 mL)	2.51	1.01	40.2	0.88	4.78
Fructose (g/100 mL)					
Before inversion	6.71	1.16	17.3	5.07	10.51
After inversion	8.03	1.18	14.7	6.38	11.76
Glucose (g/100 mL)					
Before inversion	2.39	0.643	26.9	1.23	3.99
After inversion	3.71	0.77	20.8	2.45	5.24
Fructose/glucose					
Before inversion	2.99	0.898	30.0	1.86	5.39
After inversion	2.23	0.459	20.6	1.52	3.31
Elements					
Cadmium (ppb)	4.07	3.72	91.40	1.10	19.3
Calcium (ppm)	35.44	6.69	18.88	19.70	48.70
Iron (ppm)	0.764	0.291	38.09	0.35	1.56
Lead (ppb)	26.63	15.8	59.33	11.8	86.7
Phosphorus (ppm)	149.19	52.3	35.06	73.1	289.8
Potassium (ppm)	1038.4	189.0	18.20	685.0	1365
Sodium (ppm)	19.82	4.86	24.52	14.3	38.4
Zinc (ppm)	0.307	0.105	34.20	0.15	0.54
		1980			
Ash (%)	0.210	0.0441	21.0	0.11	0.30
Brix	12.80	1.61	12.6	9.80	16.70
pH	3.69	0.581	15.7	3.23	6.54
Proline (ppm)	6.10	2.46	40.3	2.29	13.8
Specific gravity	1.0530	0.00688	0.65	1.0404	1.0705
Total acid (% as malic)	0.403	0.164	40.7	0.16	0.75
Sorbitol (g/100 mL)	0.533	0.210	39.4	0.23	1.05
Sucrose (g/100 mL)	2.64	0.947	35.9	1.38	4.32
Fructose (g/100 mL)					
Before inversion	5.33	1.08	20.3	3.00	7.92
After inversion	6.72	1.27	18.9	3.92	9.87
Glucose (g/100 mL)					
Before inversion	2.00	0.652	32.6	0.99	3.56
After inversion	3.39	0.816	24.1	2.15	5.58
Fructose/Glucose					
Before inversion	2.90	0.963	33.2	1.67	5.50
After inversion	2.04	0.426	20.9	1.43	3.20
Elements					
Cadmium (ppb)	2.816	1.42	50.43	1.30	83.0
Calcium (ppm)	37.59	9.18	24.42	23.20	63.40

Table 3. (cont'd)

Iron (ppm)	0.845	0.528	62.49	0.28	2.67
Lead (ppb)	26.42	12.2	46.18	12.60	6.15
Phosphorus (ppm)	148.87	57.2	38.42	29.2	270.6
Potassium (ppm)	1021.3	163.0	15.96	740	1350
Sodium (ppm)	19.28	4.15	21.53	13.5	28.8
Zinc (ppm)	0.301	0.0732	24.32	0.20	0.50
		1981			
Ash (%)	0.21	0.0342	16.3	0.14	0.29
Brix	12.83	1.34	10.4	10.7	15.6
pH	3.72	0.267	7.2	3.35	4.24
Proline (ppm)	5.97	2.38	39.9	2.88	12.65
Specific gravity	1.0530	0.00565	0.54	1.0442	1.0641
Total acid (% as malic)	0.441	0.183	41.5	0.19	0.91
Sorbitol (g/100 mL)	0.475	0.153	32.2	0.23	0.88
Sucrose (g/100 mL)	2.89	1.07	37.0	1.26	5.62
Fructose (g/100 mL)					
Before inversion	5.32	0.781	14.7	3.95	7.44
After inversion	6.83	0.914	13.4	5.18	9.29
Glucose (g/100 mL)					
Before inversion	1.83	0.601	32.8	0.89	3.18
After inversion	3.35	0.665	19.9	2.05	4.84
Fructose/glucose					
Before inversion	3.20	1.11	34.7	1.67	6.09
After inversion	2.09	0.372	17.8	1.48	3.21
Elements					
Cadmium (ppb)	11.77	4.46	37.89	4.70	29.1
Calcium (ppm)	42.81	9.38	21.91	25.70	61.20
Iron (ppm)	1.677	0.441	26.30	1.14	3.72
Lead (ppb)	47.91	36.3	75.77	12.8	163.5
Phosphorus (ppm)	78.02	28.8	36.91	34.7	144.9
Potassium (ppm)	1159.4	185.0	15.96	850	1510
Sodium (ppm)	23.30	7.14	30.64	16.8	53.3
Zinc (ppm)	0.508	0.177	34.84	0.27	1.06

Table 4. Mean, standard deviation, coefficient of variation, minimum, and maximum of composite 3-year sampling

Attribute	Mean	SD	CV, %	Min.	Max.
		1979–1981			
Ash (%)	0.207	0.0413	20.0	0.11	0.30
Brix	12.74	1.60	12.6	9.8	16.9
pH	3.69	0.393	10.7	3.23	6.54
Proline (ppm)	5.47	2.78	50.8	1.27	13.8
Specific gravity	1.0511	0.00702	0.67	1.0372	1.0705
Total acid (% as malic)	0.417	0.172	41.2	0.15	0.91
Sorbitol (g/100 mL)	0.524	0.214	40.8	0.16	1.20
Sucrose (g/100 mL)	2.68	1.01	37.7	0.88	5.62
Fructose (g/100 mL)					
Before inversion	5.79	1.20	20.7	3.00	10.50
After inversion	7.20	1.27	17.8	3.92	11.76
Glucose (g/100 mL)					
Before inversion	2.07	0.668	32.3	0.89	3.99
After inversion	3.48	0.765	22.0	2.05	5.58
Fructose/glucose					
Before inversion	3.03	0.993	32.8	1.67	6.09
After inversion	2.12	0.424	20.0	1.43	3.31
Elements					
Cadmium (ppb)	6.22	5.24	84.24	1.10	29.1
Calcium (ppm)	38.61	8.97	23.23	19.70	63.40
Iron (ppm)	1.095	0.595	54.34	0.28	3.72
Lead (ppb)	33.65	25.8	76.67	11.8	163.5
Phosphorus (ppm)	125.36	58.0	46.27	29.2	289.8
Potassium (ppm)	1073.0	188.0	17.52	685	1510
Sodium (ppm)	20.80	5.76	27.69	13.5	53.3
Zinc (ppm)	0.372	0.158	42.47	0.150	1.06

strength juice and preliminary studies on concentrate, it was observed that the concentrate had a lower sucrose concentration than one would normally expect for single strength juice. Furthermore, if the single strength juice was analyzed and then the analysis was repeated 6–9 months later, the sucrose concentration decreased while the glucose and fructose concentrations increased. This is the result of the hydrolysis of the sucrose to its basic monosaccharides, glucose and fructose. This hydrolysis could affect the fructose:glucose ratio. The increase in the fructose and glucose concentration would result in a lower value and a reported ratio would depend on the storage conditions of the product.

To overcome this variability and negate the effect of the ratio change over a period of time, hydrolysis of sucrose was proposed, and the fructose:glucose ratio was calculated following this hydrolysis. The data in Tables 3 and 4 indicate that this procedure reduced the range of the ratio and also reduced the coefficient of variation by approximately one-third.

Acknowledgments

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METHOD PERFORMANCE

Detection and Determination of Error in Analytical Methodology. Part I. In the Method Verification Program

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A total method verification program is presented, composed of the initial developing laboratory's method validation process and the end user's method evaluation studies. Both processes escalate in a cyclic fashion and the communicative pathway is the method transfer process. The participating laboratories acquire data by the intralaboratory procedures described and the cross-over studies entail the interlaboratory (collaborative) procedures described. During the entire process, the diagnostic techniques described result in the detection and determination of error in the methodology.

Part I

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1.0 Introduction

A method is either accurate—or it is inaccurate. This categorical statement is correct because of the now generally accepted technical definition of the term accuracy (1–9). Except for an indication of degree, nothing more or less is required. Accuracy in this text means the total error of a method, procedure, or measurement system including thereby both its systematic (bias) and random (precision) component errors.

A measurement system has a certain in situ sensitivity and specificity. (See Wilson, 8, 10–12 for definitions and discussion of these and other analytical terms.) The resultant sensitivity and specificity of a method is the sum total of the respective sensitivity and specificity contributions imparted by each of the procedural steps in the method, with the main contributions being the least sensitive or nonspecific steps, respectively. When a method whose overall sensitivity and specificity has been accepted is to be tested for its validity, the question which remains is—How accurate is it? Accuracy, unlike the sensitivity and specificity performance characteristics, is not fixed but rather is a variable because of the nature of the random error component (10, 13). Therefore, it must be measured in the hands of the persons and in the environment (place and apparatus) where it is to be used.

This operation of method verification invokes both the processes of method validation and method evaluation (14). A method validation program provides the answer to the question: How accurate is it?—and the answer is limited to that determined by the method developer in his hands. A method evaluation program provides the answer to the question: How good (i.e., how precise) is the method in the end user's hands? Almost invariably, the process of method evaluation involves a physical method transfer mechanism in that the end user is in a different laboratory. The most common example of the method evaluation process is a collaborative study (15).

In situations where responsibilities are fixed, and to attain maximum efficiency, these processes functionally should not unduly overlap. Thus, in industry, a method development group issues methods and determines their validity. The user laboratories evaluate the method's suitability in their hands. However, where organizational lines are not drawn, such as when a scientist must verify a literature or regulatory method, one needs answers to both of the above questions and, in addition, a substantiation of the sensitivity and specificity performance characteristics as well.

In essence then, when the performance of a method is either unknown or is to be challenged, a total method verification program is required. However, when the method is part of a chain in a well disciplined, functional development system, as a research and development/quality assurance operation should be, then the subdivision into method validation and method evaluation functions should be effective and efficient.

2.0 The Process of Method Development

The detection of errors and elimination of the identified error sources is a continuous process throughout the development of a method, starting with the basic measurement response system and working back to the actual sample. This process provides the transformation mechanism, with or without sample cleanup, with either dilution or concentration, from sample to analyte read-out. Automated sample and data handling may be added to the basic method core. All these steps have their individual error sources and require careful application of state-of-the-art technology and consideration of the basic scientific disciplines. In the process, the degree of specificity is locked in, and is contributed by the response system, or by the functional group or enzymatic reaction employed, or from physical separation procedures. It may result from any of these singly or from more than one additively.

Analogously, the bias component of the accuracy of the method is also built in because the resultant biases in the method depend on the zeal with which they were eliminated by design or detected and eliminated during method development (16, 17). The precision component of accuracy arises in large measure from the type of response system selected and on the overall sequential procedures composing the method. Horwitz (18), dealing with this latter point, presented experimental data from AOAC collaborative studies which show that for every 100-fold decrease in the concentration of the analyte, the relative standard deviation (i.e., CV) increases by 2-fold. This trend also has been shown by Kennedy (19), but has been generally only intuitively known. Normally, as the concentration of analyte decreases, methodology becomes increasingly complex due to cleanup and concentration steps. However, if a very sensitive response system can be employed in a simple dilute-and-inject method, relatively low

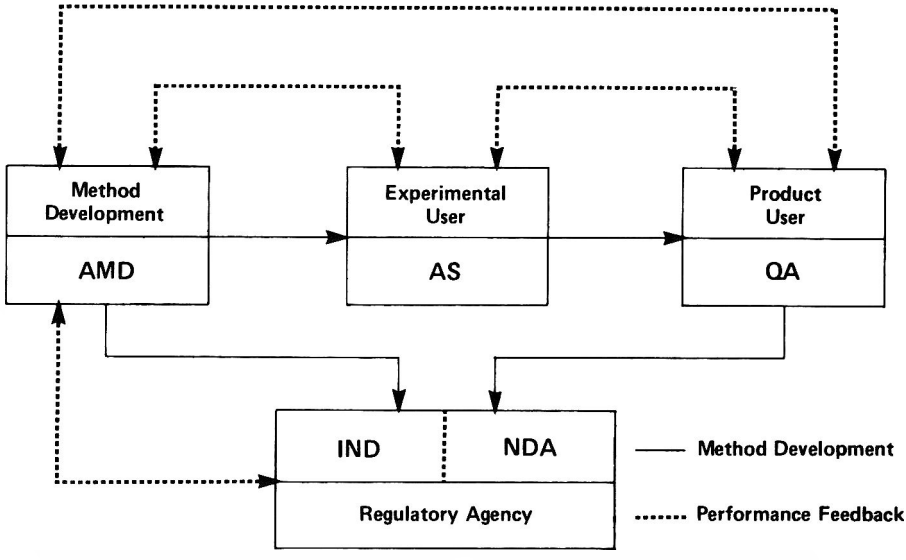

Figure 1. Method verification program in the author's laboratory: AMD = Analytical Method Development Section; AS = Analytical Services Section; QA = Quality Assurance Section.

levels of analyte sometimes may be determined with the same order of precision as are the more normal analyte levels with less sensitive detectors.

The final output is the development of a method with a status, quoting Dols (1): "(a) the method satisfies the defined needs; (b) a linear calibration function is obtained where the slope of the line defines the sensitivity of the method; (c) the method has been optimized . . . (16); (d) the method is in statistical control, i.e., all the causes of error remain the same; and (e) a set of adequate instructions defining the method has been written."

At this point the method is ready for final testing as to its accuracy; this is the method verification program.

3.0 Method Verification Program

3.1 Method Transfer

The method verification program starts with the final issue but, as shown in Figure 1, it does not end with the completion of the method validation by the method development group. Rather, method verification is a dynamic program of continuing method development from the contributions by the method users throughout the life of the product as a consequence of their method evaluation programs. Thus, at Norwich Eaton, the method is used by the Analytical Services Section to discharge its functions on experimental products, and its method evaluation program at the least provides input for optimization of certain of the practical performance characteristics (20), such as ruggedness, cost, and speed. Quite often, the reliability performance characteristics (1, 10, 20, 21) (sensitivity (linearity), specificity, and accuracy) are stressed by the differences in environmental conditions, execution, apparatus, reagents, and so on, and bias errors previously undetected become evident. These problems are either resolved or the method is returned for further modification if the effort becomes significant. The same process occurs when the product becomes marketable and the method moves into Quality Assurance. All input information on any method is coordinated for documentation and approval by the Analytical Method Development Section and is experimentally verified or amplified when necessary. As shown, further input may be received from the regulatory agency which may also perform a method evaluation. This route, however, is kept to the irreducible minimum for obvious reasons and, in practice, such an internal 2-laboratory method evaluation program is very effective for our internal purposes.

A further source of input for the continuing method verification program comes from the stability programs monitored by the experimental user. The specificity of any method, particularly one designed to be stability-indicating, is only as good as the last sample analyzed. A method may fail on any aged sample

presented and the specificity of a method is only reasonably assured after the completion of several stability programs. Consequently, a good recommended practice is for aged samples to be examined periodically by the method development group during the initial stability programs, concurrently with the regular monitoring by the stability laboratory. As will be discussed later, specificity failure is a direct interference bias error and can only be discerned by direct experimental examination techniques. There are no statistical diagnostics possible nor are there any calculational corrective measures possible.

3.2 Method Validation

The validation of analytical methods continues to be the subject of considerable discussion (22–26). The approaches have been limited largely to the validation of the final developed method from the original developer, as shown in Figure 1. All of the approaches concur on the issue of generality as contrasted with hard-lined rules, since there are many possible variations to method validation on basic considerations such as which method performance characteristics need be covered and to what extent. Some questions on which characteristics to cover have been decided by the issue of the Good Manufacturing Practices/Good Laboratory Practices (GMP/GLP) regulations. In an interesting aside, Pasteelnick (23) mentions methods that have been used for a number of years but have never been formally validated. Documentation data showing a state of statistical control on samples produced under GMP controlled process conditions should in a broadly accepted view constitute a prima facie defensible validation argument.

More directly, there is general agreement as to the method performance characteristics which are to be addressed so that the GMP/GLP regulations are satisfied (24, 27). In the original method development, sensitivity (linearity) and specificity performance characteristics will have been duly considered in the design of the method (27). Direct specificity challenge tests on the final method may be carried out by spiking the analysis samples with the suspect interferents, singly and additively. In addition, samples should be stressed under controlled environmental conditions of time, temperature, humidity, and various radiant energy exposures, such as sunlight, ultraviolet light, and incandescent light. These forcibly degraded samples will then be monitored for the intact drug content and examined using the proper detection methodology for degradants. In the case of stability-indicating assay methods, the analyte peak (and internal standard peak, if one is used) should be tested for peak homogeneity.

In the continuing method verification program, naturally aged samples should be examined by the same techniques as those developed for forcibly aged samples. Any degradants detected must be shown not to interfere in the method or suitable revision should be undertaken.

The range and the scope (22) performance characteristics must be provided for in the original development design plan, and the requirements satisfied by the final method. Process control methods must meet a previously defined range capability, usually 75–125% of the nominal analyte dosage level, for specification and quality assurance (QA) monitoring purposes. Stability-indicating methods perforce must extend to levels even lower, 40 or even 25%, to permit full-ranged scientific, artificially induced stability studies to be conducted.

It is recommended that the linear dynamic range of the standard curve be as extended as possible so that the applicability of the method can be varied by a simple selection of sample size, dilution, and response system sensitivity parameters. Methods with an extended range capability should be checked for homoscedasticity (constancy of the precision) over the actual range applied to a dosage form (22). There are two practical ways to accomplish this: 1) Compare the standard deviations of the sample range subsets (for example, 75–100–125% of nominal), each in turn, the low vs the nominal and the high vs the nominal using the F-test (28) for variances; or 2) compare the relative standard deviation (RSD) of the ungrouped assay data against the % variation [(std error of est./$\bar{y}$)(100)] as obtained from the Youden regression analysis. Does $\sigma^2 = \sigma^2_{y.x}$ (29)? See Figure 2 and sec. 3.4.4(c). If the values are unacceptably different, that is to say, for example, that the difference value is more than 25% of the RSD value of the data pack at the nominal concentration level, then the system is likely heteroscedastic and the straight line parameters should be obtained by a weighted regression technique (29, 30). The precision of the system is then only that provided by the RSD at the prescribed nominal concentration to be used for the samples.

Methods should possess as wide a scope as possible and be applicable to samples of similar nature. The validation, however, is only good for those matrixes specifically defined and tested.

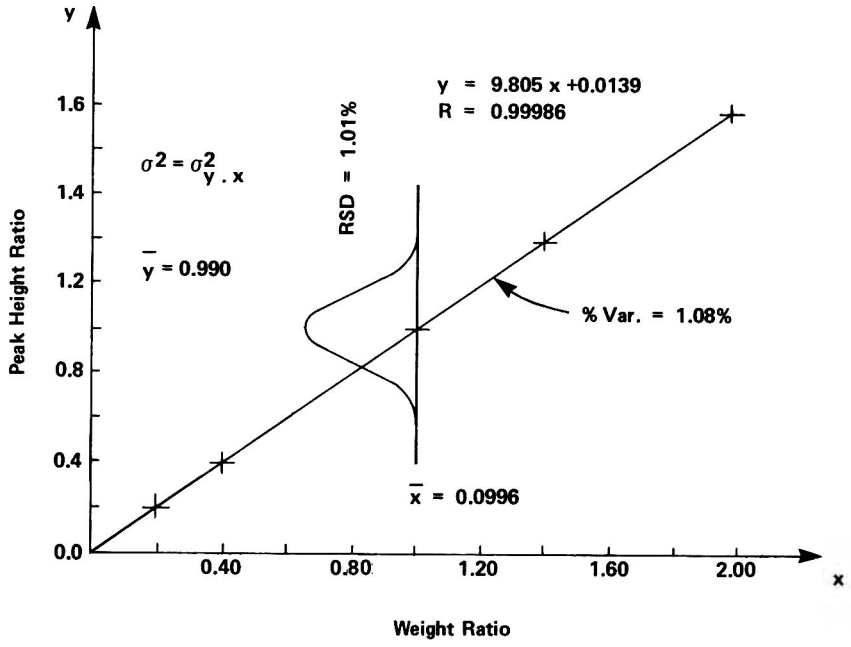

Figure 2. Linearity and precision of response curve.

Reformulated dosage forms constitute a change in the nature of the matrix and require revalidation. The amount of revalidation, however, is a judgment process depending on the extent of the matrix change, but conversely, a simplification of a formulation, as, for example, the deletion of one or more excipients seldom will require revalidation.

3.2.1 Ruggedness Test

Before a method development program should be considered complete, the effect of variables in the method, which although considered insignificant or at the least not critical, should be tested. Changes should be of the magnitude that would normally be expected to occur in the daily operations in the laboratory. Some variables are concentrations of reagents; reaction temperature; reaction time; different reagent lots (purity); types of mixing, such as manual, swirling, sonication; ambient temperature, humidity, or lighting conditions, or any other variable that can be specified.

Important variables critical to the method should be studied in detail, e.g., reaction time in a functional group reaction method, or the selectivity performance of purportedly the same HPLC columns (27). However, many variables or factors which cannot be justifiably tested individually can be tested simultaneously by using Youden's ruggedness test (15, 17), where in one plan 7 factors can be studied by performing only 8 determinations. This plan has been discussed by Hendrix (31), and an interesting and recommended half-normal plotting technique is described (32). The benefit derived from the ruggedness test is a better insight into the method variables which can make the method transfer process smoother.

3.2.2 Determination of Accuracy of Final Method

The general approaches to validating a method for accuracy have been outlined by Vanderweilen and Hardwidge (25), Letterman (24), Brewer (22), and Debesis et al. (27). It is now a generally accepted practice to test the accuracy of a method across the range of possible assay values (24, 25, 27, 33–35).

(*a*) *Systematic Error Determination.*—(*1*) *Placebo Analysis.* Certain types of samples, of which pharmaceutical dosage forms are just one example, permit the matrix of the sample to be simulated by a laboratory or actual scale preparation procedure with all the excipients present in their normal ratios except the analyte of interest. In certain other cases, the sample may be a synthetic mixture of complex natural substances that has been processed analyte-free, such as an animal feed, or naturally occurring complex substances

such as analyte-free body fluids from unmedicated patients. In any case, when such a placebo or control sample is available, it can be a valuable aid for the detection and elimination of a matrix interferent bias error in a method validation program (24).

A normal size placebo sample should be analyzed by the method and if the response level is unacceptable, the analysis should be repeated using placebo sample sizes varied over the range of possible assay values.

If the response is essentially constant, it is a matrix blank, but if it is a simple function of sample size then it is a matrix interferent, albeit low, and possibly the analyte itself. Individual analysis of the excipients can be undertaken to isolate the source of the bias. Any method that would require a separately determined matrix blank correction would normally be unacceptable because of the problems associated with the ready availability of the analyte-free material (placebo) (22). In some cases, such a method has temporary value in in-house restricted usage, pending development of a more suitable method.

(2) *Spiked Placebos.* The determination of biases may be accomplished through a recovery determination (24, 25) using spiked placebos over the analyte range of interest, for example, 75–125% of the expected (label or theory) assay value, holding the placebo constant at the nominal level. If a matrix blank was observed, and particularly if it appeared to be variable with placebo sample size, the analyte spike to placebo ratio should be alternately varied by holding the analyte spike concentration at the nominal level and varying the placebo size over the range of interest. Although the ratios of analyte to placebo are about the same as those in the reverse procedure above, the actual sample sizes approximate more closely those in actual analyses, and a direct observation of the matrix interferent is possible without knowing its nature. Moreover, this variation can eliminate the need for the variable size placebo interferent study mentioned in the preceding section. If the interferent substance has been identified, it should be directly tested (24) for its interference by direct addition to a nominal size placebo in an amount which produces a response 1×, 2×, and 5× that of the matrix blank response observed.

A statistical analysis of a found vs taken function is then made (36, 37). The value of the slope of the regression line is compared against the expected value of 1.0 and similarly, the intercept value from the regression line is compared against the expected value of zero. If the confidence interval of the slope calculated using the selected confidence level, alpha, conventionally 0.05, includes the value of 1.0 and if the confidence interval of the intercept includes the value of zero, then the statistical inference may be drawn, respectively, that the slope value is not significantly different than 1.0, and that the intercept value is not significantly different than zero. In other words, in the case of the slope, no indication of a proportional bias error is evident, and in the case of the intercept, there is no indication of a constant bias error.

Should a statistical significance be found, then the converse inferences are drawn. If the respective indicated biases are unacceptable, further direct experimental examination of the method for the bias sources and their elimination is required.

Another diagnostic technique which is commonly applied to such data is to group the recovery data into range subsets and then calculate the mean ($\bar{x}$) and the standard deviation (s) of each subset. The subsets are then subjected to the conventional t-test for means, the 75 vs the 100% and the 125 vs the 100% to discern whether there is any statistical difference in the subset means. If there is, a shortening of the linear dynamic range in the assay practice is required or, if that is unacceptable, further method bias studies are required. If there is no statistical difference, the $\bar{x}$ and s values of the ungrouped data are calculated.

Recovery bias is calculated from the general expression:

Bias = found − (added + placebo blank)

and the calculation can be made for the mean observed difference at any one subset range, or the mean observed difference averaged over the entire range (ungrouped data). The confidence interval for the bias is then the average found minus the effective average added $\pm ts/\sqrt{n}$ where t is the Student's t-value for the chosen confidence level (usually 95%) at $n - 1$ degrees of freedom, and n is the number of assay tests in either the subset or in the total ungrouped set (28).

An alternative technique for calculating the spiked recovery data has been presented by Vanderweilen and Hardwidge (25) who use the fundamental relationship (38):

total error (accuracy) = bias error (systematic) + standard error (random).

The first term in their equation 2 is the relative total error and the second term is the standard error (39) so that the difference is the desired

relative bias error. Unfortunately, they set the value inflexibly at 1.5% without indicating any regard for the end purpose of the method under development. The bias specification also should be plus or minus because it can vary in either direction. In any case, this alternative is the mathematical equivalent of the previous one and offers no advantage but rather, arbitrarily, attempts to set a limit for acceptable bias which can only be made by the end user for the specific case.

The acceptance criterion for bias recommended by Debesis et al. (27) is subject to the same criticism of inflexibility and has also been challenged as being overconservative (40).

(3) *Alternative Approaches.* If a placebo cannot be obtained as is quite often the case, there are several other options available. One option which Youden (41) and Wilson (12) both consider is the comparison of experimental results with those obtained on a standard reference material (SRM), such as the SRMs that are available from the National Bureau of Standards (NBS) (42). More directly, the SRM can be used to prepare the standard curve when the matrix of the samples is known to be completely related, or where comparison of the experimental analysis data against that for the SRM shows the results to be in acceptable agreement.

The bias is calculated as before except that the difference is between the found assay results and the known SRM value. Again, the confidence interval is evaluated as before, $\pm ts/\sqrt{n}$ (12).

A second option is the comparison of the sample experimental results with those obtained by a standard method, preferably one which has been shown to be free of bias error (43, 44). In this instance, the reference method is taken as possessing zero bias error. Then, the bias is equal to the mean of the method under examination minus the mean of the reference method. Wilson (12) and Natrella (45) give the detailed expressions for the calculation of the confidence interval of the bias for the more complex situation where the methods have unequal variances (see Section 3.5.1 for the equal variance case). In the pharmaceutical industry, a common use of this approach is the comparison of a developed method against a compendial method which is legally defined as an unbiased reference method. Here, the developed method may be the preferred method. For a complete validation procedure under the legally defined stipulation, see Section 3.5.2, Greenbriar procedure.

A third option (already referred to in section 2.0) is to conduct a detailed "first principles" analysis of the individual steps in the procedure for the respective bias error in each step (38). Then, the total bias error is the sum of the individual steps. This reference should be consulted for details; however, the basic requirement is that the chemical measurement process (i.e., the method) must be in a state of statistical control if the bias errors are to be assessed. This approach is analogous to that used at NBS in their SRM program as one of the ways by which they arrive at an analyte concentration value along with the indicated variance range (42, 46). Complex SRMs, such as the various steels, rubbers, plastics, etc., have no accurately known value except for the manufacturing or synthesis expected value, hence the NBS procedure for establishing an *absolute* concentration value is the *definitive* exercise in the detection, elimination, and/or correction of bias error in the chemical measurement process. These procedures deserve careful study for analogous application to the world of industrial, environmental, or biological samples.

A fourth option for establishing the true value of the sample so that the bias of the method can be stated is to compare the results of the method on a sample by using one or more different analytical methods (1, 41). Each alternative method should be based on quite different "first principles" and each method should be replicated sufficiently (perhaps 20 to 30 in-day repeatability values) on the sample to determine a reliable standard error (random) value. If the results agree, the absence of bias error is not unequivocally proven but there is strong evidence that bias error is not present. The conviction increases with the number of methods used. Again, this is another avenue used in the NBS SRM program (42, 46). If the results disagree, one or all of the methods could be inaccurate and conclusions must be carefully drawn.

A fifth option, principal component analysis, is a combination of the approaches used in the 2nd and 4th options above (47). In this approach, quite different methods are used, no less than 4 or 5, and none of the methods should be significantly inferior to the others. Rather than choosing or relying on a single reference method, principal component analysis uses the results of the several methods to provide a composite reference value, in essence compensating for the lack of rigor of single methods by pooling the several methods. The main limitation of the approach obviously is the availability of the different methods.

A sixth option is use of the Greenbriar proce-

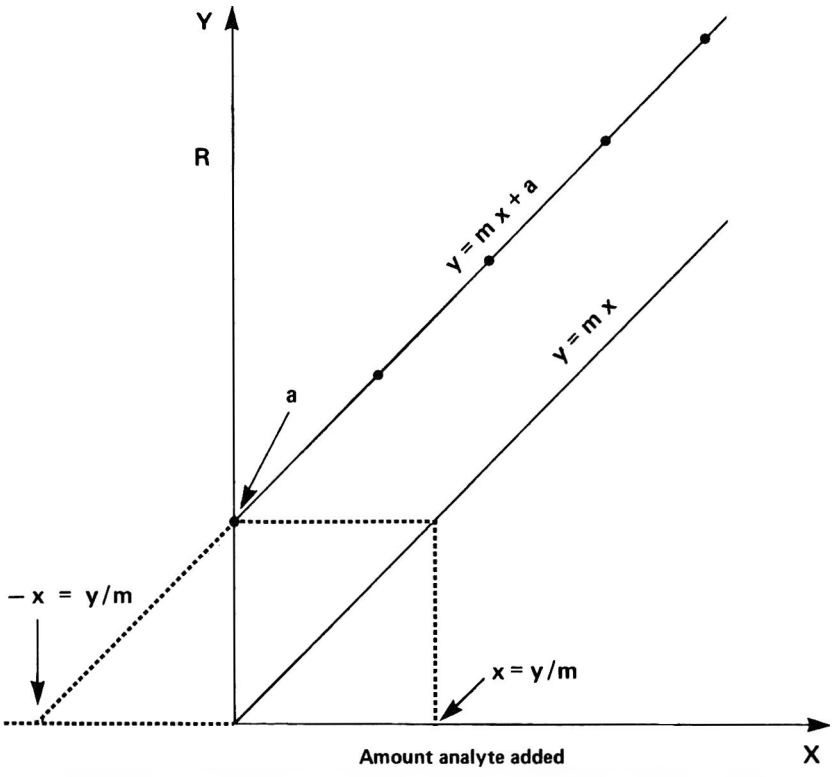

Figure 3. Plot of data for method of standard additions: linear response.

dure, Section 3.5.2.

(4) *Method of Standard Additions.* Of the above techniques for determining systematic error, the method of standard additions is one of the most important. This technique has been variously called method of additions, incremental addition method, autostandardization method, standard addition method, method of successive addition, and mixture method. Chow and Thompson (48, 49) applied the misnomer internal standard method.

In addition to using the method of standard additions to determine bias in a method by spiking placebos as already discussed, which Skogerboe and Koirtyohann (50) refer to as a spike-recovery study, the method of standard additions may be used in the analysis of real samples to overcome calibration and matrix interaction biases (51, 52), and by proper diagnosis of the data, the technique is valuable for the detection and elimination of some bias error sources. The standard addition is always made to an actual sample matrix, whether the matrix analyte content is zero, as for a placebo, very low as in trace analysis, or at nominal concentrations, as in environmental analysis or in pharmaceutical dosage forms. The principles and techniques remain the same. Calculations are made either in terms of recovery of the addition or an assay of the unspiked sample.

The technique is performed by adding either a small amount of a standard to the sample, or a small amount of a standard solution to an aliquot of a sample solution, and in both cases completing the analysis with and without the addition(s).

The data are plotted as shown in Figure 3. The unspiked sample is shown on the ordinate as the intercept a whose concentration is to be determined, and it is seen that when $x = 0$ (i.e., no spike addition), $y = a$. The concentration of analyte in a, that is, the value of x, may be determined in 2 ways. The first and most commonly employed is to calculate the abscissa intercept value from the equation of the experimentally determined additions line, $y = mx + a$ for the condition where $y = 0$ which is $-y/m$ as shown. (The minus sign is physically meaningless.) The value of a $(=y)$ to be used should be the intercept as obtained from the regression analysis and not the experimentally determined value (34). The second and simplest way is to

use the regression equation, $y = mx$ (as determined for the condition where $a = 0$). In either case, the unknown analyte concentration x in the unspiked sample is equal to the ratio of the unspiked sample response to the slope of the additions line.

An important requirement in the technique is that all solutions, unspiked sample, and spiked samples, be diluted to the same final volume so that any interferent present will be present in the same concentration in all solutions, and will equally affect the analyte originally present or incrementally added (53). Some consideration should be given to the nature of the analyte moiety in the unspiked sample solution and to adding the same analyte moiety in the spike, or providing the proper solution chemistry, e.g., pH, so that the same moiety is present. Failure in this regard is one of the principal criticisms of the technique (50). A second criticism by Skogerboe and Koirtyohann (50) is that spike addition changes the ratio of analyte to interferent and may result in a change in the interaction effect (54); however, they acknowledge that observed linearity of the system preempts this failure. Thus, linearity over the range of incremental response, 2× to 4× the unspiked concentration with 2 or more additions is a requirement for validity.

Another requirement (55–58) is correction for background (system blank) to avoid high results. The correction is made by using the best conventional practice for measurement of the blank for the particular technique, e.g., in flame spectroscopy, the luminosity of a water blank was determined (58).

The question of blank correction arises in an interesting way in a variation of the method of standard additions where it has been implied to be a *multiple* additions technique. The use of multiple additions is mandatory to demonstrate linearity of the system over the dynamic range of interest. However, once linearity is known, a novel single addition variation has been recommended by Dean (59) and Menis and Rains (51). Their single addition technique is *not* to be confused with a single addition value calculated from the basic proportionality, $C/C + S = R_1/R_2$ (60), where C is the unknown analyte concentration, S is the analyte standard addition, and R_1 and R_2 are the unspiked sample and spiked sample responses, respectively. This expression leads to $C = S(R_1/R_2 - R_1)$, the conventional expression used by many workers (53, 61, 62). Willis (62), in applying this expression, erroneously stated that a blank correction is not necessary. Dean (59) and Menis and Rains (51) incorporate the use of a standard curve by interpolating the R_1 and R_2 responses from the standard curve to obtain the corresponding analyte values x and y. In their expression, $C = X(S/Y - X)$, the term $S/Y - X$ is a correction factor which should be between 0.5 and 1.5 to avoid erratic results.

It is obvious that by use of the standard curve, the calculation above automatically incorporates the system blank from the standard curve and that a simple subtraction of the standard curve system blank from the R_1 and R_2 observed responses would accomplish the same end result. However, the unique advantage of the calculation is in the correction term which is a valuable *diagnostic* device, because deviations from unity are indicative of the magnitude and direction of the bias errors.

The principal operational defect of the technique is that even if the above requirements are met, the standard additions technique may only be effective in eliminating biases from the point at which the spike is made. Prior steps in the sample handling procedure may still be a source of bias. For this reason, the spike addition point must be chosen as close to the original sample as possible. With special care, the spike addition can be made to the original sample (60, 62–65), and this was in fact the technique described by Foster et al. (61, 66) in what apparently are the first applications of the method of standard additions described in the literature. The results from this approach may then be compared with the results from spike additions farther downstream. If they are essentially the same, then a more convenient later point can be used.

Any substance which acts as a direct interference in the method of additions will cause a biased result because the technique requires that the response be due only to the specified analyte and nothing else. Certain other types of interferences discussed by Klein and Hach (67) can render the technique inapplicable, such as interferents which can react (complex) with the analyte, cause reagent depletion or reagent inefficacy, or matrix imbalances, such as pH or buffer capacity.

Although McBryde (68) has shown that the technique is not effective for the purpose of improving the precision of the response (absorbance) measured on the unspiked sample by bringing it from below the level for minimum error (69), as in a trace analysis application, up to a value corresponding to the level of minimum analysis error, there are optimum values for the

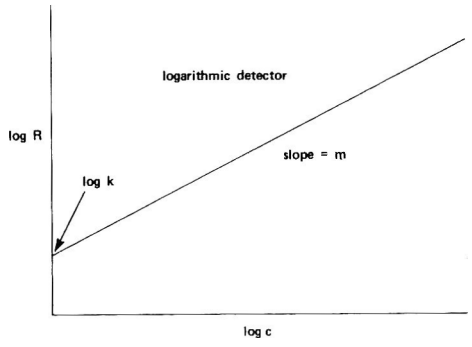

Figure 4. Plot of data for method of standard additions: logarithmic response.

response measurements in the technique. Most workers (51, 52, 56, 60, 63, 64) state that the optimum values for the additions are equal to, twice, or 4 times the level of analyte in the unspiked sample. Beukelman and Lord (52) have provided an error function analysis for flame spectrometry, as have Meites (65, 70) and Reinmuth (71) for polarography. Meites (65) recommends the maximum level of the addition to be just below C_{max}, the point at which the wave height of the analyte no longer is proportional to its concentration.

Larsen et al. (55) present a discussion of the uncertainty (imprecision) of the standard additions line using regression analysis, while Franke et al. (72) discuss the optimization of system parameters.

In an approach called generalized standard addition method, Saxberg and Kowalski (73) present a mathematical model for the analysis of multicomponent mixtures in which the principal advantage is that the response of each analyte need not be fully selective, as is required in the conventional single analyte method. The generalized method provides a means of detecting and quantitating interference effects, and simultaneously determining analyte concentrations. Both linear and nonlinear models are considered but no experimental data are given.

The case of nonlinear response, such as is seen when using ion selective electrodes or as seen in flame spectrometry, has been discussed in detail by Beukelman and Lord (52) and Nagy (74). Here the response may be logarithmic, quadratic, or other mathematical function of concentration, c, namely: $R = kc^m$. This function yields a plot as shown in Figure 4.

Transformation of the data, either by using appropriate instrumentation or mathematically, may yield a resultant linearity which satisfies the requirements. (This is analogous to the transformation of transmittance data to absorbance through the relationship, $A = -\log T$.) If the untransformed data must be used (52, 60, 63, 74), the calculation becomes inconvenient and somewhat complicated, requiring iteration.

(*b*) *Random Error Determination.*—(*1*) *Precision Measures.* The randomness, that is, variability of a method, may be quantitatively estimated by a number, a statistic called standard deviation, s. This capability unfortunately is not possible with the other reliability method performance characteristics. It is easily determined and its measure is made at various stages of the method verification program. The earliest determination of s, in a modified form, has been mentioned in Section 3.2, in connection with the method performance characteristic range, and is illustrated in Figure 2. The standard deviation about the standard curve is estimated by a statistic called the standard error of estimate, $S_{y\cdot x}$, which expresses numerically the imprecision of the line, that is, the variability of the standard curve data points. This statistic when divided by $\bar{y}$ (mean of the responses) and multiplied by 100 yields % variation (*not* variance), another statistic comparable to the familiar relative standard deviation (also called, coefficient of variation, CV). Since the variability of the standard curve represents a significant portion of the total method variability, this statistic is a good early method development estimator. Unless the method is a dilute-and-inject type where most of the method variability is in the standard curve, the value of % variation should be less than one-half the total method imprecision target value, otherwise there may be a precision acceptability failure in the final method when it is applied to samples over the required range (27).

(*2*) *Measurement.* The precision of the final method should always be determined on actual samples, production samples in the case of manufactured articles such as dosage forms. In early method development before these are available, simulated samples such as spiked placebos may be used, but the results are only for scientific information and are of limited value in documenting true method precision (24). If the method under study is to be used as a QA control method, fresh production samples should be obtained. For a stability-indicating method, however, the most naturally aged samples available should be used. The conventional procedure is to obtain a set or subsets of analysis results on a homogeneous, stable sample at the analyte concentration values of interest and

calculate the relative standard deviation of the set or subsets. As was described above for determining method bias (Section 3.2.2), most workers now generally test the precision of the method across the range of possible assay values, the range depending on whether the method is for production control or for use in monitoring stability programs.

Again, the results are evaluated as previously described for the determination of bias by using spiked placebos. The subset values are tested for a hypothesized non-variation in the means by the *t*-test. If no significant difference of the means between the range subsets with the nominal value subset is found, the standard deviation of the entire set of data is calculated and reported as the standard deviation of the method. If an unacceptable difference is found, the presence of a bias error is indicated. A search for the error source should be made. See also Part II for a possible corrigible error correction. Since the concentration of analyte in real samples is unknown, a correlation plot of found values vs true values is not possible.

In addition, several problems arise in the practice of determining these performance characteristics. Some of these are: how many analyses on each sample overall; how many on each subset; should all values be taken in one day (repeatability) or all again on different days (reproducibility); how many different days; how many analysts; how many laboratories?

For the final method in the case where the method is the first involving the analyte in the particular matrix, 5 results at each subset range per day by one average analyst over two different days is considered adequate (22–24). The average analyst is a trained analyst familiar with the method; the different days should be as different as possible (3 to 5 days apart); and all reagents, instrument settings, standard solutions, and other operations should be completely renewed and repeated.

If several analyte levels are involved, the above is performed on the one that has the highest matrix-to-analyte ratio and the others are tested at only one range level, with at least 3 results on one day by one analyst. Different analyte levels and matrices will require sound professional judgment as to how much cutback can be enjoyed.

Many of these options will be reconsidered in the discussion of method evaluation (Section 3.3).

(3) *Priority.* The question arises during method validation as to which should be studied first, the bias of the method, or the precision. Westgard and Hunt (75) state that they study precision experimentally first "because systematic errors are difficult to evaluate when large random errors are present," and this is most emphatically correct. On the other hand, of what value is it to run a precision study of the scope described above and then have the data defaulted by a subsequent discovery of an unacceptable bias error?

The most common experience in method development is that the bias and precision studies are an iteration and escalation process with the precision first estimated from the standard curve % variation statistic, then by a recovery study from either a spiked placebo or a method of standard additions study. In any case, none of these early studies should be elaborate, but rather exploratory. After several trials (modifying along the way), if the method seems to be viable, a short version precision study is indicated, usually 3 to 5 results at the nominal level by one analyst in one day. If these results are acceptable, the ruggedness test is performed, changes are made as indicated, and then the final accuracy study should be made. If the method remains fundamentally intact, the final precision study can be made with some assurance of its acceptability.

(4) *Objective.* Vanderweilen and Hardwidge (25) and Debesis et al. (27) considered the end use precision requirements of a method during the method development stage. The acceptability of the method precision depends on its standard deviation, the range of the probable product specifications, and the acceptability of the number of assay replications to be required. These criteria, either as possible limitations or trade-offs, should be part of the method development objectives as influenced by proper input from the end users.

The desired precision of the results can be achieved either by developing a method with the required precision for a single result or by one which must be statistically assisted through replication. If a method is to be used on a one-time basis, or for a low-volume application, a relatively imprecise method that would require more replicates could be an economic trade-off for the longer method development time that would be required for a more precise method. Conversely, a method precise enough to permit use of single result assays would be worth the method development time for a valuable high volume or high cost product.

These considerations bring into play 3 statis-

tical terms, all of which utilize the $\bar{x}$, n, and s values as reported in the final precision statement for the method, namely, the confidence interval (CI), the prediction interval (PI), and the tolerance interval (TI):

$$CI = \bar{x} \pm t_{1-\alpha/2,n-1}\, s/\sqrt{n}$$

$$PI = \bar{x} \pm t_{1-\alpha/2,n-1}\, s(1 + 1/\sqrt{n})$$

$$TI = \bar{x} \pm ks$$

where k is a tabular value using P, the fraction of the measurements to be included in the interval and the confidence coefficient $\gamma(1 - \alpha)$.

(*c*) *Accuracy of Methods for Bulk Drugs.*—As Vanderweilen and Hardwidge (25) have indicated, a bulk drug assay is usually an assessment of purity. Most assays are comparative and are simply a ratio of the response of the bulk drug lot to that of the analytical reference standard. In this type of analysis, any bias errors are normalized and so, by definition, the accuracy statement is simply the precision statement.

If a reference standard is not yet available, the method can be compared with an independent method. (Spiking techniques are inapplicable.) Such an independent method should be chosen to use quite different first principles, or it can be a recognized, validated method, or a compendial method. However, the best option, if available, is the use of an absolute method (such as titrimetry, gravimetry, or quantitative nuclear magnetic resonance analysis). Such an absolute method may already be in hand from the analytical reference standard program.

Since most comparative bulk drug lot assays have inaccuracies in the range of greater than 0.5% and some as high as 2%, their scientific value is questionable in a modern program of GMPs, process validation, specific identity (characterization) tests, and a protocol of other purity index tests. An analytical chemist can appreciate the value of determining the iron content of an alloy steel where the iron content may be a minor component, but does one determine the iron content of a mild carbon steel where the iron content is 99+%! It is and has been axiomatic that impurities are more accurately determined directly *if* and *where* indicated. The best indicators for these come from quantitative techniques such as thermal analysis and phase solubility analysis which yield the averaged impurities content without possessing prior knowledge of their individual nature or number (76).

(*d*) *System Suitability.*—Since the concept of the test of a chromatographic system for suitability was first described (77), amplified (78), and adopted by the U.S. Pharmacopeial Convention (USP) (79), the system suitability test has become a standard part of the method validation program. Debesis et al. (27) have most recently described the test in detail in complete agreement with USP XX. They point out the difference between the one-time determination of the necessary chromatographic parameters *during* method development and the daily check against those test parameter specifications to ensure the viability of the chromatographic system.

There is no disagreement on the chromatographic parameters tested, relative retention, α; capacity factor, k'; column efficiency, n; resolution, R; and tailing factor, T, as described by the USP (79) with R and T generally recognized as the most important. However, the author agrees with Roman (80) that the precision measure (repeatability) portion of the USP test (27, 79) should be included as part of the method reference standard procedure rather than as a separate test, and Roman's discussion deserves study.

Palermo (40) has pointed out that systems other than chromatographic must also demonstrate on a viability basis that the specificity requirements for the end purpose of the method are met.

The system suitability test is one of the principal techniques available to any laboratory, especially quality assurance, for the purpose of meeting the requirement of maintaining methodology in a state of statistical control.

3.3 Method Evaluation

The answer to the question of how good, that is, how precise, is the method has been answered by the developing laboratory. An analytical chemist could not competently issue a method without having satisfied his own standards that the objectives had been met. Nonetheless, a formal method evaluation procedure is necessary, which subjects the method to use acceptability and demonstrates the required precision in the end-user's hands. The method is of value only if it is found to be usable and to meet the precision requirement. For maximum efficiency and effectiveness in the method verification program, the "direct testing" procedure should be restricted to real sample analyses only. Direct bias error studies are accepted as claimed in the method description. Part of the method evaluation program involves the application of recognized diagnostic tests which serve as bias error detectors, and the results of these tests are handled via the feedback mechanism referred to

previously in Figure 1. Also, any method in use provides its own read-out indicators which can signal any anomalous response from prior samples, and these also trigger the feedback mechanism. Thus, the user is a vital part of the overall bias detection capability provided by the method verification program.

3.3.1 Determination of Acceptability of Final Method

(*a*) *Method Transfer Procedure.*—The analyst in the evaluating laboratory makes an initial run-through of the method, preferably on the same sample used in the developing laboratory for the final precision study. In this run-through, any difficulties in the interpretation of the method description are noted as are problems due to differences in instrumentation and other laboratory practices requiring adjustments not intended as changes in the method. These points may be discussed with the method developer, or perhaps personally observed in the evaluating laboratory by the method developer. Any practical suggestions for resolving the problems imposed by the laboratory differences should be made and accepted by the method developer. Youden (15, 90) has discussed these initial problems in connection with interlaboratory testing. The objective is to arrive at a clearly written method which both or all laboratories can understand and execute in a comparable manner.

The formal interlaboratory test data can then be obtained. The sample must now be the same as used in the developing laboratory. If this is not possible, or if the method has undergone revision, the developing laboratory would be required to generate data on the same sample to be used in the evaluation study since the purpose is to demonstrate that equivalent results can be obtained in the accepting laboratory. The number of results to be obtained and the number of range subsets is a matter of judgment for each method. Usually on first-of-the-line methods, 5 results at each of the usual 75–100–125% of nominal subsets is adequate for both control and stability-indicating methods. Later, dosages of different strengths, different dosage forms, or reformulated dosages can all be studied by an appropriate abridged program, as for example, 5 results at the nominal level only. In-day variability comparison is desirable because the objective is to determine agreement between laboratories, and not the variability of the method within the laboratory. (This is a matter of concern at another time. See next section.)

Table 1. Precision by lot pairs: across lots

Lot	Assay Day	mg/g		d	d^2
1	1,6	312.00	315.22	3.22	10.37
2	2,6	312.20	315.66	3.46	11.97
3	6,7	328.59	330.39	1.80	3.24
4	6,12	328.05	333.99	5.94	35.28
5	1,13	318.78	328.68	9.90	98.01
≈	≈	≈	≈	≈	≈
29	3,15	327.31	328.64	1.33	1.77
30	3,9	329.20	330.24	1.04	1.08

n = 30 pairs $s = \sqrt{\frac{\Sigma d^2}{2n}} = 3.73$ $\Sigma d^2 = 835.44$

Lot 29

n = 30 $s = \sqrt{\frac{\Sigma(X - \bar{X})^2}{n - 1}} = 4.48$

$F = (4.48)^2/(3.73)^2 = 1.44$ $F_{0.95}(29,29) = 1.85$

The data for each range set or subset are compared via the *t*-test for means and the *F*-test for variances. The comparison can be made within the subsets to discern whether a trend is present and between the sets or subsets of the 2 laboratories to test for agreement.

If the data fail to pass the statistical tests, and further minor modifications in the method agreeable to both participants cannot resolve the problem, the method is returned for further method development.

If the data pass the acceptance criteria, the method becomes official as written and the total data generated in the interlaboratory study should be used as the method performance precision statement. This is not so for an abridged study, however, where the data may not be as extensive as the method validation precision study.

(*b*) *Other Techniques for Determination of Precision.*—(*1*) *Duplicate Pairs.* Duplicate pairs of data may be easily accumulated in a quality control laboratory by the simple expedient of rerunning one of the samples of a set with a later set. The periods may be regular or irregular. Table 1 shows partial data for 30 lots of samples. Lot 1 was run on the first day and then that same sample was rerun on the sixth day; lot 5 was also

Table 2. Pooling of variances: single dose level, stability program data – initial station assays

Lot No.	n	$\bar{X}$ (%)	s
1	12	2.294	0.0450
2	4	2.323	0.0232
3	7	2.255	0.0029
4	8	2.220	0.0206
5	2	2.271	0.0297
6	4	2.310	0.0163
7	4	2.284	0.0173
8	4	2.238	0.0142

k = 8 N = 45 $\bar{X}_w$ = 2.272 S_p = 0.0314

s, from individual observations (n = 45)

s = 0.0447

Table 3. Pooling of variances: multiple dosage levels, stability program data – initial station assays

Lot No.	n	$\bar{X}$ (mg/g)	s	RSD
1	5	915.0	18.125	1.981
2	8	915.1	12.112	1.324
3	9	778.9	7.390	0.949
4	8	777.8	4.097	0.527
5	5	520.2	3.962	0.762
6	5	919.4	11.546	1.256

k = 6 N = 40 RSD_p = 1.13%

N – k = 34

run on day 1 and then rerun 13 days later. As many data pairs per set may be run as desired but the minimum would be one pair generated per set. Each pair must be runs from different days. The standard deviation for the data pairs is calculated from the expression, $s = (\Sigma d^2/2n)^{1/2}$, and is shown in Table 1. As an illustration of the validity of the procedure, one of the lots (lot 29) was run on 15 different days in duplicate each day and the usual ungrouped standard deviation was calculated. Between-days data is required for this comparison because the pairs technique is a between-day precision mode. The results indicate that the standard deviations by the 2 approaches are not statistically different.

Several advantages (15, 21, 82) for the QA laboratory accrue with this procedure: 1) The lot samples need only be stable enough for the period selected for the pairs determination; 2) the procedure is valid across lots and the precision measured is independent of lot-to-lot variation; 3) the standard deviation measured is one of the best long-term *in usage* measures of the precision of the method, and is the value that is intended to appear as the *usage* precision statement in the method description; and finally, (4) the standard deviation may be used for QA control charting purposes.

(2) *Pooling of Variances.* Whenever archival data are being accumulated as in stability programs, the initial assay data for each lot represent an opportunity to apply the statistical technique for pooling variances. These data occur in 2 types, the first, which we now consider, where the stability lots are at the nominally same analyte level. In this case, the value of the standard deviation for each lot is calculated as shown in Table 2 and then pooled. The expressions for pooling are:

$$\bar{x}_w = \frac{n_1\bar{x}_1 + n_2\bar{x}_2 + \ldots . n_k\bar{x}_k}{n_1 + n_2 \ldots . n_k}$$

$$n_1 + n_2 \ldots . n_k = N$$

$$S_p = \sqrt{\frac{[(n_1 - 1)(s_1)^2 + (n_2 - 1)(s_2)^2 + \ldots .(n_k - 1)(s_k)^2]}{(n_1 - 1) + (n_2 - 1) \ldots . (n_k - 1)}}$$

$$- 1 + n_2 - 1 + \ldots . n_k - 1 = N - k]$$

where $\bar{x}_w$ is the weighted grand average and S_p is the pooled standard deviation (9), N is the number of total assays, n_i (i = 1,k) is the number of assays in the ith lot, and k is the number of lots. Again, S_p is independent of the lot-to-lot variation, so this measure of precision is again a means of ascertaining whether the method is in a state of statistical control. If the data in Table 2 were incorrectly calculated in the usual way from the 45 values accumulated in the 8 lots, a value of 0.0447 would be obtained for the standard deviation. This is a significant 42% difference due to the lot-to-lot variation in the ungrouped standard deviation figure. Again, each S_p value should be reported and incorporated into the method description as the *usage* precision statement.

The second type occurs when the lots on stability taken are from multiple dosage (analyte) level forms (Table 3). In this case, the precision

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measure is the relative standard deviation, RSD, rather than the standard deviation, to normalize the analyte level. The rest of the calculation is the same; the RSD values are used in place of the s value in the equation for S_p.

The RSD_p value is a measure of the overall method precision with little or no contribution from product variation (i.e., lot to lot and dosage level). If confidence intervals are desired, the values for the corresponding level must be calculated using the actual standard deviation determined for that level.

(3) *Youden Plot.* Because the Youden plot is primarily a diagnostic test for detecting bias error, the discussion of the measurement of precision from the Youden plot data is reserved for Section 3.4.3 (b), Diagnostic Tests.

(4) *Greenbriar Procedure.* Part of the output of the Greenbriar procedure is the precision measure of the method. See Section 3.5.2.

3.4 Diagnostic Tests

The diagnostic tests to be discussed in this section are those that use real sample data using the final written method. (We have already referred to several diagnostic test inferences that occur during the method development process.)

These free-standing planned diagnostic tests may be used by any of the participants in the method verification program, and are of special value in difficult method transfer situations. However, their continuing value is as part of a QA protocol to ensure that methodology is in a state of statistical control (2,3,83). Note that this QA function is parallel to, but distinctly different from, the more familiar product specification/process control QA function.

Because a method is composed of many operations in a myriad of possible environmental conditions, the situation posits a total variance from these factors which may be individually understood in general but which cannot be specifically identified and controlled. Hence, this total variance must be accepted as the method performance after the optimization process has been completed. The purpose of the ruggedness test (Section 3.2.1) is to eliminate most of the individual laboratory variations of ill defined factors in a method executed as written. Even so, the data from a diagnostic test are intended to help elucidate any such factors that still may be present.

These components of variability represent 2 distinct types of randomness, repeatability and reproducibility (84). The repeatability/reproducibility ratio of a data set [or batch (8)] may in one case be a within-laboratory/between-laboratory variability as in a collaborative test, or in another case, a within-day/between-day single-laboratory variability (85). Repeatability, an approximation of the true randomness component of variance where the factors at play cannot (in a practical method) be further controlled, arises from sources of error in the operation of the method and is common to all laboratories. Reproducibility, a systematic component due to factors which could practically be still further controlled if pinpointed, arises from determinate sources of error such as the following: a newly introduced but somewhat more impure reagent; the introduction or use of a different instrument in the laboratory, or the difference in instrumentation between laboratories; differences in calibration arising from the reference standard, handling, and storage or somewhat different measurement techniques in the calibration procedure; and subtle-to-substantial methodology variations in the laboratory practice.

It must be kept in mind that whenever any statistical test is employed to detect a true difference, that the discriminatory power of the test depends in a crucial way on the number of observations or samples taken. With too few samples, "equivalent" results may only reflect the inability to effectively discriminate due to the large variance of the test and not to the absence of a significant difference.

3.4.1 Repeatability/Reproducibility Ratio

The absolute value of the repeatability/reproducibility ratio is not a constant of the method procedural system (82, 86, 87) but is variable even in a single laboratory and in fact can vary from one set of measurements to another. This is nothing more than the old familiar problem of establishing the characteristics of the periodicity of the set stability and therefore the establishment of the frequency for running standards interspersed with samples (recalibration). In essence then, we have a randomness component which can vary from set to set and a systematic component which can also vary from set to set due to the particular and changing combinations of the factors of which each is composed. This is the reason a method must be monitored constantly during its usage in a QA program, and why it must be assured to be in a state of statistical control.

The significance of the repeatability/reproducibility ratio has been discussed by Youden (15) as mentioned in Section 3.4.3 (b) but more

fully by Horwitz (18, 88). The values reported by Horwitz (88) are the coefficient of variation (%) for repeatability and reproducibility (CV = RSD) to normalize the varying concentration of analytes across the different methods. The normal ratio is given as $^1/_2$ to $^2/_3$, that is, the repeatability RSD is 0.50 to 0.67 of the reproducibility RSD. When the repeatability RSD is very close to the reproducibility RSD, that is, the ratio is greater than 0.7, the laboratory is performing consistently (within-day, single-laboratory study), or all laboratories are performing consistently (within-laboratory, collaborative study). The random component is the major source of variance, and if the absolute total variance is acceptable, the method is satisfactory. A repeatability RSD much less than the reproducibility RSD, that is, a ratio less than 0.3, means that the variability is high on different days (or between laboratories) due to the many possible laboratory operational factors already mentioned. The source of the reproducibility variance should be sought.

3.4.2 Ranking Test

A diagnostic test devised by Youden for comparing measurement data by collaborating laboratories (89) has been applied to analysis of data from collaborative tests (15, 90). The test data are examined to see if any laboratory consistently obtains high or low values. For m laboratories, a rank score of 1 is given to the laboratory with the highest result, 2 to the next, and so on until the mth laboratory is reached. If more than one level (sample) is being tested, each laboratory rank score across all the samples is totaled. These rank totals are compared with the range of values in a distribution table for m labs and k samples (Table B, p. 85, ref. 15), which should include the observed rank score. If a rank sum score falls outside the tabular limits, there is only a 1 in 20 probability that it is due to chance alone and the greater probability is indicative of a laboratory-biased result. If the errant score is consistently high (or low) for all samples, the evidence becomes unmistakable. This test is useful for eliminating such laboratories from a study. A fuller discussion of this diagnostic test is available (91).

3.4.3 Interlaboratory (Collaborative) Tests

The purpose of a collaborative test is to determine if the method description is clear and complete and as a part of the method transfer process, that it does yield equivalent results in user laboratories. In effect such a study is a diagnostic procedure because it is intended to pinpoint the presence of possible bias errors.

In a study such as those conducted by AOAC (88), the larger the number of collaborative laboratories the better, because a wide range of environments and conditions is desirable. Differences in interpretation and execution of the procedure, particularly any large method departures, whether deliberate or unwitting become obvious sources of systematic error. A simple 2-laboratory study plan using only one sample has already been described as the routine transfer process in Section 3.3.1(a).

Collaborative studies are of 2 principal types and both are described in detail in the AOAC statistical manual (15). Part II of the manual, by Steiner, presents the conventional, and perhaps to collaborators outside AOAC, the more familiar type based on a statistical analysis of variance (ANOVA) diagnosis of the data (82, 84, 86, 92–95). The other approach, Part I, by Youden, which was the original manual, is perhaps the most familiar to AOAC collaborators. It is a unique 2-sample procedure and has most recently been described by Filliben (96). Both types require the same number of assays.

(*a*) *Analysis of Variance Procedure.*—In the ANOVA procedure, the choice of the number of samples k depends on the range of interest, invariably the nominal level, and usually the 75 and 125% levels, $k = 3$. The number of laboratories is designated as m and the number of replicates r, usually $r = 2$. Each laboratory receives the 3 samples, which are analyzed in duplicate by the same average well trained analyst, preferably following a blind sample coding so that duplicates are not run sequentially. The data are collected by the study coordinator and analyzed statistically. Steiner (15) gives a detailed example for the model where $k = 3$, $m = 10$, and $r = 2$. For this model, the ANOVA provides estimates for the various components of the total variance: between laboratories (reproducibility), between replicates (within laboratories or repeatability), and laboratory-sample interaction. The laboratory-sample interaction component measures the extent to which the laboratories do not obtain consistent differences among the sample levels. If a significant laboratory-sample interaction is seen, a search for the cause should be made to determine why the laboratories handle the different level samples in nonparallel ways. If such a search is not deemed necessary, the data should be recalculated for each level separately as indicated below for the single sample abridged model. Moreover, it should be kept in mind that

a nonsignificant laboratory-sample interaction term does not mean that an effect was not present, but rather that none was detected by the test.

If only one sample is available, then only the between-laboratories and between-replicates (within-laboratories) variance component sources can be estimated. If duplicates are not determined, the between-replicates variance component cannot be estimated. Steiner also gives the mathematical treatment for these model abridgements.

The principal advantage of the ANOVA procedure is that all the determinations from the range levels, k, are calculated interdependently providing the laboratory-sample interaction estimate. As we shall see later, the Youden 2-sample approach can only study one range level at a time. The ANOVA procedure makes 3 assumptions: 1) that the overall errors are normally distributed, 2) that the errors are statistically independent, and 3) that the errors have the same variance. Connor (93) has discussed the validity of these underlying ANOVA assumptions.

(*b*) *Youden Two-Sample Technique.*—In Youden's technique (17, 81, 90, 97), 2 similar samples are selected, varying somewhat in analyte concentration but of the same general matrix characteristics. The 2-sample data pairs are generated by having each laboratory analyze each sample *once* after practicing if necessary on a different sample, and the data are collected centrally and tabulated as shown in Table 4, each laboratory with its pair of values, X and Y. The difference and total columns are as shown. In the difference value, any systematic bias error drops out because it is in each measurement, but in the total it is doubled. A standard deviation calculation of the 2 respective columns would yield the same result if no systematic bias errors were involved, but would be statistically different otherwise. Therefore the standard deviation is calculated for the differences and the sums but these standard deviations need to be corrected by dividing the corresponding s values by $\sqrt{2}$ because 2 measurements are involved for each datum point. The corrected difference standard deviation s_r is the value for the between-laboratories random error component while the corrected sums standard deviation s_T is the total bias error. By using the additive property of variances, $V = s^2$, it is a simple matter to calculate the systematic between-laboratories bias error s_B for the Table 4 data, and to show that the random bias error is 35% of the total bias error, a value

Table 4. Youden plot data: single determination on 2 materials by 25 laboratories

Lab	Sample X	Sample Y	X − Y (d)	X + Y (t)
1	0.31	0.22	0.09	0.53
2	0.08	0.12	−0.04	0.20
3	0.24	0.14	0.10	0.38
≈	≈	≈	≈	≈
25	0.14	0.10	0.04	0.24

$\bar{X} = 0.229\ \bar{Y} = 0.134\ \bar{d} = 0.095\ \bar{t} = 0.363$
(algebraic)

$\bar{d} = \bar{X} - \bar{Y}$
$\bar{t} = \bar{X} + \bar{Y}$

$s_d = 0.0695\ s_t = 0.114$

$s_r = s_d/\sqrt{2} = 0.048$ $\qquad s_T = s_t/\sqrt{2} = 0.081$

$s_B = \sqrt{(S_T)^2 - (S_r)^2} = 0.065$

Reference: W. J. Youden, in H. H. Ku, NBS Spec. Pub. 300, Vol. 1, p. 136, Table 1

consistent with a general observation from collaborative studies that systematic errors are most always the predominant factor (15). This calculation is more relevant than an F-test calculation on the 2 variances, s_T and s_r, which can be done but which would indicate only the statistical significance of the difference in values and not a percentage as above.

(*1*) *Youden Plot.* The ingenuity of the Youden 2-sample diagnostic test is seen in the graphical presentation of the data in Table 4 because each datum point in the plot from the pair of data from each participant is evaluated on an individual identity basis. In contrast with the ANOVA technique, as is typical of statistical calculations, the members of a population lose their identity. Both techniques will, as stated by Horwitz in the preface to the AOAC manual (15), yield the same quantitative estimates of the between-laboratory and within-laboratory bias errors, but only the Youden plot can yield the detail for the individual participant.

The Youden plot (Figure 5) is prepared by plotting the data pair from each laboratory in the conventional fashion as an X,Y coordinate point.

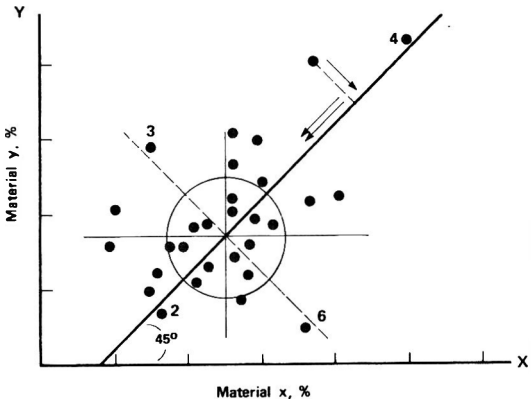


Figure 5. Youden plot: collaborative 2-sample chart.

The scale of the abscissa and ordinate axes must be the same and chosen to include the ranges of all results reported. There will be a point for each laboratory and this point can be plotted using an identifying code, e.g., a symbol, color, or number. Lines parallel to the X-axis and Y-axis, through the $\overline{X},\overline{Y}$ coordinate point are then drawn, which divides the plot into 4 quadrants. A line 45° to the X-axis through the $\overline{X},\overline{Y}$ coordinate point is drawn next, and last, a circle of radius $s_r b$ is drawn with the $\overline{X},\overline{Y}$ coordinate point as the center. The radius of the circle to contain the percentage of the points desired if the individual systematic errors could be eliminated is calculated by multiplying the random standard deviation s_r by a factor b. This factor, in turn, relates to the percentage of points to be contained in the circle by the expression $b = \sqrt{-2\ln(1 - \%/100)}$ as derived from the equation given by Youden (81). This formula comes from a circular bivariate normal distribution where the X,Y data pairs are taken to be statistically independent and have the same variance. The value of b for 95% of the points to be included is 2.45, and a table of b values is given (81) or may be calculated by using the formula given above.

The diagnosis and interpretation of the Youden plot is fairly straightforward but a careful reading of Youden's own original explanations (17, 81, 90, 97), which are all collected in Ku's compilation (28), is strongly recommended. A brief description follows. If only random effects were at play, the data for both materials would be scattered in all possible combinations of high and low values for materials X and Y, respectively; thus, both could be high, both low, and one high–one low, and vice versa. In such a situation, the points would be clustered around the central point of the circle and 95% of the points (2σ) would be included within the circle, randomly distributed in the 4 quadrants. In this ideal situation, there would be no evidence of a between-laboratory bias, only a within-laboratory random bias. The more likely event is that a laboratory which gets a high result on one material would be likely to get a high result on the other material also; similarly, a low result on one material is most likely to be matched closely with a low result for the other. The effect is that for whatever causes are responsible for the systematic bias above the random level expected (i.e., points outside the circle), the points elongate into the upper right and lower left quadrants, in other words, along the 45° line. It is also evident that if the results on both materials were both high or both low by the same relative amounts, they would fall exactly on the 45° line, meaning only systematic error is present. It follows then that the distance a point is off the 45° line, measured by a perpendicular from the point to the line, is a measure of the imprecision (Figure 5, single arrow). Also, the systematic error is measured by the distance along the 45° line from the foot of the perpendicular to the central point (Figure 5, double arrows).

Thus, at a glance, the point for any one laboratory discloses whether it has an imprecision problem, i.e., the inability to repeat itself, as shown in Figure 5 for both laboratories 3 and 6. The former obtains a high result on material Y whereas the latter obtains a high result on material X. On the other hand, laboratories 2 and 4 are able to reproduce the results on the 2 materials closely, but laboratory 2 is consistently low whereas laboratory 4 is consistently high. The other laboratories that fall outside the circle have a problem with both precision and systematic errors. (Figure 5 is the Youden plot from ref. 81 altered to amplify the above remarks.)

By an examination of the plot, the direction for improvement can become clearly evident. Thus, a long narrow ellipse of points along the 45° line indicates that each laboratory is very precise but that they are using their own versions of the method so that a more careful rewrite is required. On the other hand, points considerably out on the 45° line (2 and 4) could mean that the laboratory has made a substantial change in the method. The imprecision noted for laboratories 3 and 6 could simply be due to a change in analysts on the pair of samples, a simple error in execution, an error in calculation, a mistake in typing, or other similar explanations.

The disadvantage of the Youden plot com-

pared with the ANOVA procedure is that only one sample pair/level can be studied at a time. Youden (15) recommends 3 pairs (or unit blocks) representing the high, middle, and low range of amount present, and this entails the same number of samples as an ANOVA for the 3 range samples; however, the Youden plot technique does not provide for a laboratory–sample interaction estimate. Horwitz (15) states in the preface to the AOAC manual that although the 2 techniques are different, and an acceptable choice for either may be made depending on the views of the users, the results by both approaches agree. The Youden plot will appeal to the analytical chemist for its simplicity and visual impact, and the ANOVA approach to a statistician-oriented laboratory.

(2) *Youden Plot Heterogeneity Test.* In a modified form of the 2-sample plot, Youden (81) has described a means for checking lack of heterogeneity in the 2 similar samples. In the diagram, if the distribution of points is roughly circular but the diameter of the circle of points is too high, the cause may be high imprecision or heterogeneous samples, and no discrimination can be made from the plot. By carefully preparing double-size samples and splitting these between coded pairs of laboratories, the usual plot then allows this discrimination to be made, because if heterogeneity is the problem, the results from the coded laboratories will be seen as doublets, whereas if imprecision only is the factor, the usual random spread in the data points will be seen. In addition, if doublets are seen, the spatial distribution can also indicate whether only one of the samples was heterogenous.

(3) *Youden Plot with Additional Paired Samples.* In most cases where the Youden plot technique could be used, the number of participating laboratories will be rather small, possibly only 5 or less. To accumulate more points, each laboratory should analyze additional related pairs of samples. A Youden plot is prepared for each pair as previously described with the $\overline{X}$ and $\overline{Y}$ lines. The plots are now superimposed, matching the central points of each chart and the lines. All of the points are now transferred to one chart with one pair of lines, and the 45° line is drawn in on the composite chart. Each pair of samples should be coded to be able to identify the laboratory for the respective paired-data point. If a laboratory is able to repeat itself accurately, the additional data-pair points will overlap. The degree with which they do not overlap provides further insight into the within-laboratory reproducibility.

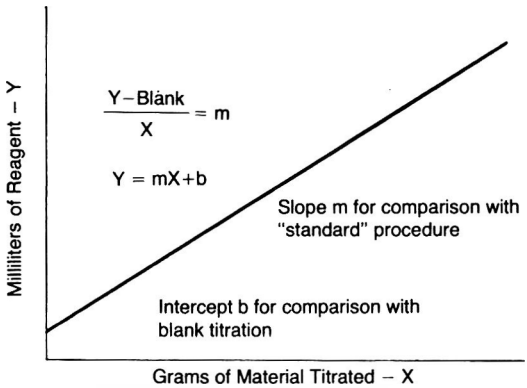
3.4.4 Intralaboratory Procedures

(*a*) *ANOVA Procedure.*—In the collaborative tests (Section 3.4.3), the repeatability/reproducibility ratio was determined in both the replication/multiple sample ANOVA procedure, and in the Youden 2-sample procedure. The reproducibility determination in the form of the between-laboratories estimate of variance is no doubt the maximum stress situation for the variable systematic error component, but if a method is to be used across a broad spectrum of users, as is the case with compendial methods, this is probably what is desired. However, a more general situation is a method with fewer users. In this case, an ANOVA model would provide for the determination of the ratio in a single laboratory by the method developer before release, or by a user upon receipt.

Wilson (8) has discussed such a model where, using the same notation as in Section 3.4.3(b), k samples representing the range levels as before are analyzed in duplicate ($r = 2$) on p days. Wilson uses the term batch for a set of runs but the concept is the same, namely, that a set or batch is that period of time during which the calibration curve remains constant. To ensure that the data are statistically independent, the samples should be coded and randomized before analysis. The ANOVA data collection and calculations are the same with the following variance component estimates: between days (reproducibility), between replicates (repeatability), and sample–day interaction. The ANOVA plan can be found in any statistics text (45, 98), but for convenience, by substituting *day* for *laboratory*, the model detailed by Steiner (15) can be followed.

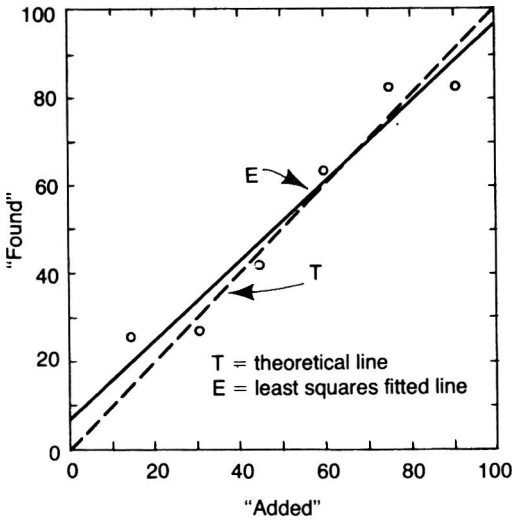
(*b*) *Youden–King Two-Sample Procedure.*—The modification of the Youden procedure from an interlaboratory to an intralaboratory procedure was made by King (85) in an analogous manner as described for the ANOVA procedure above. The pair of similar samples are analyzed once per day over a period of 15 days. King found the technique useful with as few as 10 sets of analyses but generally planned on 20 to 30 sets over a period of 1 to 2 months. The technique can be used to detect the systematic error between days as before for between laboratories. The between-day (reproducibility) and within-day (repeatability) standard deviations can be calculated as before, the Youden plot prepared, and the Horwitz ratios and interpretations can be determined and interpreted analogously.

(*c*) *Youden Regression Test.*—In 1947, Youden (99, 100) first reported a diagnostic technique



W. J. Youden, Anal. Chem., 19, 946 (1947); figure taken from Linnig, Mandel and Peterson, Anal. Chem., 26, 1102 (1954).

Figure 6. Youden regression line.



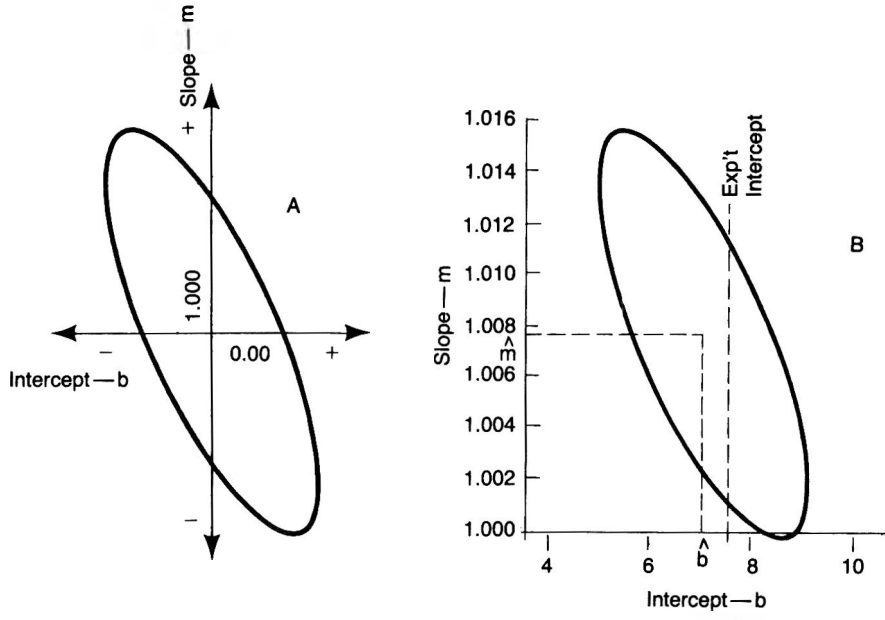
Mandel and Linnig, Anal. Chem., 29, 743 (1957).

Figure 7. Effect of experimental errors on fitted straight line.

which can be used not only to unambiguously detect the presence of a systematic bias in a measurement system, procedure, or method but also to delineate its nature and, most important, to determine it quantitatively. It is most unfortunate that Youden did not emphasize the quantitative nature of the measurement of the constant error which his technique provides. Linnig et al. (101) were the first to amplify Youden's method for the study of accuracy. These workers correctly diagnosed the presence of a constant systematic error from the observation that high "found" values decreased slowly and asymptotically as the amount of sample increased. They also related the slope of the sample size response curve, which this writer calls the Youden regression, as a reciprocal function of the proportionality constant (equivalent weight) of the analyte in the sample titrated. (In gravimetry, the slope is a reciprocal function of the gravimetric factor; in absorption spectrophotometry, it is a reciprocal function of the absorptivity, and so on for each type of responsivity.) The intercept of the Youden regression function was interpreted as a measure of a constant error but regrettably they concluded it to be a correction *to* the blank. The reverse is true; the blank is part of the intercept as determined from the Youden regression line [see Figure 6, taken from Linnig et al. (101)]. The chemical blank, or system blank as it is now more commonly called, comes from the intercept of the standard response curve. The intercept from the Youden regression line contains this same system blank plus any other analyte-matrix interaction bias contributions so that the total Youden blank (TYB) is equal to the Youden blank from the matrix plus the system blank or: $TYB = YB_M + SB$.

In 1961, Youden (41) reiterated the value of his sample size response curve as a diagnostic for constant systematic error and further showed that proportional systematic error is *not* disclosed by the technique, regardless of whether the (Youden regression) line went through the origin. Most recently, the Youden regression has been recommended by Dols (1) as the best technique for determining the intercept value to make the joint confidence ellipse diagnostic test.

(*d*) *Joint Confidence Ellipse Test.*—Lark (102), referring to Youden's papers (99, 100) on his interpretations of the intercept and slope in terms of the statistical significance to zero and unity, respectively, pointed out the strong interrelationship between the two and the erroneous conclusions which can result from treating these independently of each other. Mandel and Linnig (37) expanded on this interdependency and presented a concept due originally to Working and Hotelling in 1929 (103) called the joint confidence ellipse. Mandel and Linnig demonstrated the negative correlation of the intercept and slope as shown in Figure 7, which according to the theory of least squares is the usual situation for linear data. Due to the experimental variability of the slope and intercept values, a range of possible values exists for an experimental measurement system, and Working and Hotelling (103) showed that this confidence interval is



A. Perfect case: from regression data, slope m = 1.0 and intercept, b = 0. Experimental blank, nil.

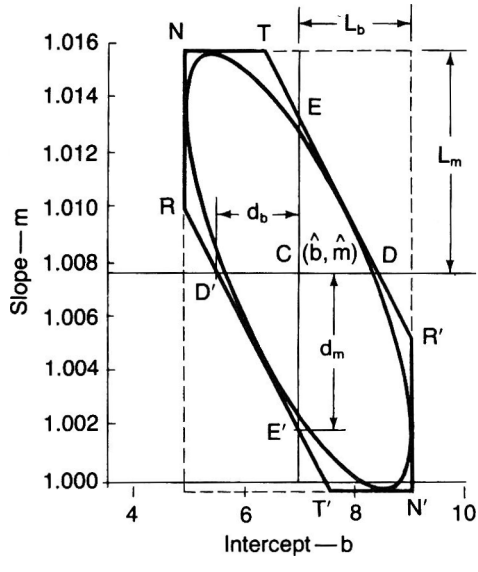
B. Example from Mandel and Linnig, Anal. Chem., *29*, 743 (1957). Regression data: m = 1.0077, b = 6.99. Experimental blank = 7.40.

Figure 8. Joint confidence ellipse for slope and intercept.

an ellipse, as shown in Figure 8. The tilt of the ellipse is due to the negative correlation effect.

To apply the diagnostic test, one simply determines the values of the slope *m* and intercept *b* as obtained from the linear regression line for the found vs added data. The system blank may be experimentally determined on a blank in the conventional manner or as the response curve intercept *to compare* against the regression line intercept, *b* value.

The details of the drawing of the ellipse (Figure 8) or a graphical approximation (Figure 9) from the statistical equations derived are found in Mandel and Linnig's paper (37); however, the calculations require only the *X,Y* analysis input data. A computer program is available (R. C. Kohberger, Lederle Laboratories, Pearl River, NY, private communication). If the values of the intercept and slope as a coordinate point fall within the ellipse, then no statistically significant bias is evident. Thus, in the example, the point *b,m* falls within the ellipse for either the Youden regression intercept or the experimentally measured value, indicating for this case that either is a valid measure of the blank. In addition, since a value of the slope above unity is required to keep the coordinate point within the ellipse,



Mandel and Linnig, Anal. Chem., *29*, 743 (1957).
Also, reprinted in Dois and Armbrecht, JAOAC, *60*, 940 (1977).

Figure 9. Graphical construction of joint confidence region.

the data suggest that in addition to the constant error indicated, a relative (proportional) error also exists.

3.5 Validation of Alternative Assay Methods

The need for validating alternative methods arises in several ways; the most common case is when a new or revised method is required by a new formulation or a reformulation. The validity of the new revised method may have been proven as described in Section 3.2.2 for the new or reformulated product, but is the method valid for all the previous formulations which coexist, e.g., samples in the field or in on-going stability programs? If possible the replacement method must be applicable to all the related products as discussed under Section 3.2, Range and Scope. The option to continue with the former method on previous formulations is always open but the cross-over to the replacement method requires a formal alternative method validation study.

Besides a change in the form of a product, the need for an alternative method can arise because of new technology permitting more ruggedness, or speed, or better accuracy for marginal methods, or a more sensitive method with a lower determination level. For all these reasons, alternative methods must be proven to be comparable to the methods they replace, in terms of the use intended. Usually, faster and more rugged methods without loss of accuracy is the predominant reason but in some critical cases, a method which is more accurate, albeit slower and perhaps less convenient, could be the desired methodology.

For the comparison study, methods must be comparable in performance capability. Thus, if a validated stability-indicating assay method is to replace a nonspecific assay in a stability program, a cross-over study is not required, and the nonspecific method is simply dropped. Conversely, for obvious reasons, a slow, inconvenient stability-indicating method cannot be replaced with a fast, simple nonspecific method.

There are many possible ways of conducting an alternative method validation study (82), all of which represent selections of the factors considered important for the particular case. In the following section are approaches based on the assumption that an alternative method has been validated, but in the Greenbriar procedure (104, 105) the validation of the alternative method and the comparison between methods are performed simultaneously. Youden (15) has also applied his 2-sample procedure in an interlaboratory study involving 2 or more methods.

Within the method verification program, 2 situations normally occur. One is a noncritical case where the alternative methodology is closely related to the method in current use, which uses well established techniques. In the other, the alternative methodology may be a relatively new technique for the laboratory, hence more data are required and the reproducibility criterion is mandated.

3.5.1 Extension of the Normal Method Development Program

(*a*) *Noncritical Applications.*—(*1*) *Comparison of Means* (*t-Test*). A typical sample representative of the nominal concentration of the product is selected for analysis by both methods. Two variations can be distinguished. In the first, the study will be completely done by a single laboratory, and hence the number of determinations on the sample by each method can be made equal, $n_1 = n_2$. In a second variation, the sample has already been run in one laboratory and is to be run in another, on the same sample, and $n_1 \neq n_2$. We do not know the variability (σ) of either method but we can assume that they are substantially the same. (Later, this assumption can be checked by the *F*-test as will be shown.) Also, since our primary concern is the equivalency of the methods, we only wish to test the within-day (repeatability) precision, so all values will be run as a set and we presume that the set of data available from the second laboratory has also been similarly obtained. The data and calculations are as shown in Table 5 (pp. 3-23, ref. 45). Since $|\overline{X}_1 - \overline{X}_2| > \mu$ (0.04 > 0.025), a significant difference is implied between the means of the 2 methods. The 95% confidence interval estimate of the true difference between the means, $m_A - m_B$ is equal to $(\overline{X}_1 - \overline{X}_2) \pm \mu$ or 0.015 to 0.065. Because this interval does not include zero, we conclude that the observed difference between the means is significant. However, the width of the interval, $2\mu = 2(0.025)$ as a fraction of the average $\overline{X}$ of the 2 methods may be acceptable on an absolute basis for the purpose and both methods may then be accepted. Also, the ratio of the variances, the *F*-test, $s_2^2/s_1^2 = 1.71$ against the tabular value of $F_{0.95}$ (7, 12) = 2.91 strengthens the validity of our assumption that these methods had equivalent variances. If these data had failed the *F*-test, an alternative calculation by Natrella for unequal variances (pp. 3-26, ref. 45) is available. Last, if $n_1 = n_2$, the equation for the confidence interval μ simplifies to:

$$\mu = t \sqrt{s_1^2/n_1 + s_2^2/n_2}$$

Table 5. Comparison of means of 2 methods: variability unknown but assumed equal (2-tailed *t*-test)

Method 1	Method 2	
79.98	80.02	
80.04	79.94	
≋	≋	$\alpha = 0.05$
		$df = n_1 + n_2 - 2 = 19$
		$t_{1-\alpha/2}(19df) = 2.093$
80.00		
80.02	79.97	

$\bar{X}_1 = 80.02$ $\bar{X}_2 = 79.98$ $\bar{X} - \bar{X}_2 = 0.04$

$n_1 = 13$ $n_2 = 8$

$s_1 = 0.0240$ $s_2 = 0.0314$

$\mu = 0.025$ $\mu = t\, s_p \sqrt{\dfrac{n_1 + n_2}{n_1 n_2}}$

$$s_p = \sqrt{\frac{(n_1-1)\, s_1^2 + (n_2-1) s_2^2}{n_1 + n_2 - 2}}$$

(2) *Paired Measurements* (*t-Test*). In some special situations, a sufficient amount of a pooled sample may not be readily available, or only at some inconvenience, e.g., blood and serum samples requiring large withdrawals, or freezing, storing and thawing. Some samples may not be stable, or can vary considerably in concentration, as for example some process intermediates. In such cases, determinations on a sample may be taken in pairs by the 2 methods and the acquisition of data pairs continued over a period of time on similar related samples. Thus, the comparison of the 2 methods is made *within* pairs, eliminating any effect of the concentration difference between the samples; yet the data are acquired under reproducibility conditions, which represents the maximum stress comparison condition.

Barnett and Youden (34) have reported on such a procedure; the data and calculations are shown in Table 6. The number of pairs is optional; at least 5 are recommended. The average difference between the pairs, $\bar{d}$, is an estimate of the difference between-methods systematic bias error, and the question becomes, is it greater than the normal random error at the degree of confidence chosen. Since $|\bar{d}| > \mu$ ($7 > 6.77$), there is a significant difference between the average paired differences, signifying a difference in the method means. The 95% confidence interval estimate of the true difference between the paired differences is equal to $\bar{d} \pm \mu$ or -13.77 to -0.23. Because this interval does not include zero, we conclude that the observed mean difference, $\bar{d}$, is significant. The width of the interval, $2\mu = 13.54$ as a fraction of the average $\bar{X}$ of the methods is also unacceptable, so that the alternative method is unacceptable by either criterion.

Table 6. Comparison of 2 methods: randomized complete-block ANOVA plan

	Method 1	Method 2
Day 1	$X_{11} \ldots X_{15}$	$Y_{11} \ldots Y_{15}$
Day 2	$X_{21} \ldots X_{25}$	$Y_{21} \ldots Y_{25}$
Day 3	$X_{31} \ldots X_{35}$	$Y_{31} \ldots Y_{35}$

(*b*) *Normal Applications.*—In this intermediate plan, we simply repeat the first day results over several different days; three is a conservative yet meaningful choice. The plan is shown in Table 6. The results are all taken on one selected sample by the 2 methods *in the same laboratory* on the different days, a variable number of results per method per day, usually at least 5. Again,

Table 7. Alternative method comparison: paired measurements, 2-tailed *t*-test

Date	Method 1	Method 2	d	
3/28	86	89	−3	
3/29	92	96	−4	$\alpha = 0.05$
4/19	92	79	+13	
≋	≋	≋	≋	$t_{1-\alpha/2}(9df) = 2.262$
4/21	86	110	−24	

$\bar{X}_1 = 90.2$ $\bar{X}_2 = 97.2$ $\bar{d} = -7$

$n_1 = 10$ $n_2 = 10$ $s_d = 9.46$

$\mu = t\, s_d/\sqrt{n}$ $\mu = (2.262)(9.46)/\sqrt{10} = 6.77$

$$s_d = \sqrt{\frac{\Sigma(d-\bar{d})^2}{n-1}}$$

the effect desired emphasizes the method performances, and an interlaboratory effect would be a needless complication. Such a plan would require 30 analyses, 15 by each method. If necessary, it could be reduced to 3 analyses per day per method for a total of 18. The ANOVA is used to provide an estimate of the between-methods variance component, between-days variance component, a method–day interaction variance component, and the total variance. The between-days estimate is not of interest in this case, but if the method-day interaction term is significant, the methods are not performing the same from day to day, and this could be a significant factor for investigation.

3.5.2 Greenbriar Procedure

The Greenbriar procedure (104, 105) is a specially designed ANOVA plan that allows the comparison of the alternative method, 2 alternative methods if desired, with the official reference method, incorporating the error sources of duplicate sample assays at 3 concentration levels on 3 different days using at least 2 different analysts on at least 2 of the days, on 6 lots of the normal production material.

The data output is intended to provide not only the between-methods variance estimate, which is the primary purpose, as for the procedures in Section 3.5.1, but also the method precision data for each procedure and the bias estimate for each procedure, constituting thereby a complete method accuracy statement. In the procedure, the provisos for duplicate assays (repeatability) and for 3 different days (reproducibility) provide the desired precision estimates of each method. The requirement to test 6 lots of material to check the variability of production material is irrelevant to the 2 expressed purposes of the study, namely, a comparison of the 2 methods and the determination of the accuracy method performance characteristic of each method. Production material variability is a different problem and its incorporation into the plan constitutes a needless and very large complicating factor in terms of the number of analyses and complexity of the study.

The bias estimates for each method are derived in either of 2 ways: 1) by the difference in the determined values against the known values, or 2) by using the average of the values determined by the reference method as the true unbiased value. The latter may be only a legal stipulation and thus scientifically questionable, and the former may be quite valid but depends on the specific nature of the sample. In the study design, consideration of sample validity is stressed by the stipulation that the material tested be as authentic as possible and not just admixtures; for example, tablets where the production material is a tablet, etc. This stipulation is, in most cases, a limiting factor in terms of economic feasibility. However, it should be noted that if a recovery study (see Section 3.2.2) were to employ a spiking technique to prepare known samples, that same experimental approximation would also be acceptable when used as part of the Greenbriar procedure.

In essence, then, the Greenbriar procedure has considerable merit as an all-inclusive procedure for both validating a method and at the same time comparing it with a reference method. There are many occasions where methodology is not developed but is available from secondary sources, and the method performance characteristics are unavailable or unknown. However, if a method is being developed, the progressive validation/evaluation/method transfer process and finally as the last step, the validation of the alternative method as described is probably the preferable pathway.

3.5.3 Youden Two-Sample Procedure for Two Methods

Youden (15) has also applied his 2 similar sample procedure for the comparison of 2 methods, but in this application the graphical technique is not as dynamic in terms of the effort expended vs the other options presented in Section 3.5.1. The approach consists of preparing a Youden plot for each method and visually comparing them. The algebraic calculations are the same as those in Table 4 for each individual method, but the F-test is applied to the ratio of the standard deviations of the differences and sums, respectively, of the 2 methods. Thus s_{d_2}/s_{d_1} and s_{T_2}/s_{T_1} are calculated and compared with the tabular value for $F_{0.05}\ df_{(n-1)(n-1)}$ for significance. Also, the t-test on the difference of the method totals, $(X + Y)_2 - (X + Y)_1 = d_T$ is applied. All of this is more complicated and no more informative than the tests in Section 3.5.1.

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Detection and Determination of Error in Analytical Methodology. Part II. Correction for Corrigible Systematic Error in the Course of Real Sample Analysis

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A new perspective in the functional operation of corrigible errors is presented. If the incorrigible errors, calibration, and direct interference are absent, the new proposed calculation procedure will provide a correct analytical result; results obtained by the method of standard additions will agree with results from the conventional standard curve technique on the same sample; and different methods will produce the same analytical result on the same sample within the variance of the random error of the method(s). The technique is also a diagnostic tool for the detection and determination of the corrigible constant and proportional errors.

Part II

1.0 Introduction

The sources of systematic errors (1, 2) present in analytical methodology may be classified into 4 categories (3): 1) calibration/system, 2) direct interference, 3) constant, 4) proportional. A further subclassification which can be made and one which is important to the objectives of this paper is that error sources 1 and 2 are separate and distinguishable from error sources 3 and 4 in that there are no statistical diagnostic tools possible for the detection of the former, and hence no calculational corrective techniques are possible. Their presence can only be discerned by direct experimental examination or from prior knowledge of the sample history. Conversely, error sources 3 and 4 can be detected by statistical diagnostic techniques (4), their magnitude can be quantitatively determined, and their values can be used for correcting analytical results.

Corrective calculations have always been possible in conventional analytical practice but these are constants that are separately determined and most often not on the actual sample for analysis, but on either a different sample, or a simulated sample. Examples are the chemical blank as an approximation of constant error, and the recovery factor as the approximation of proportional error when both use a placebo (4).

The purpose of this paper is to demonstrate that the determination of the constant and proportional errors present in an analytical method may be made as part of an actual sample analysis, that correction of the analytical results leads to the correct analytical value within the variance of the method if error sources 1 and 2 are not present, and that results from different methods will agree if this condition is satisfied for each method.

2.0 The Nature of Systematic Errors

2.1 Incorrigible Errors

Any error in the calibration process, either in the reference standard or in the measurement system, will cause a systematic error in the sample analysis results. For example, if the reference standard through careless or improper handling were allowed to sorb moisture, results calculated against such a standard would be biased high in proportion to the sorbed water

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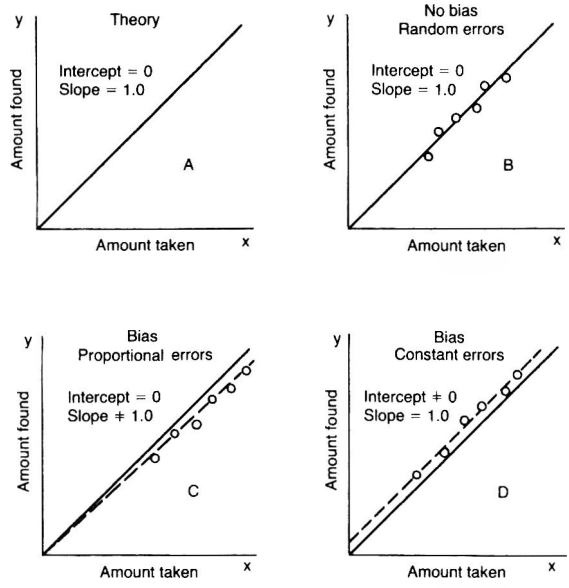

Figure 1. Characterization of corrigible errors (Figure 1, modified, from Youden, W. J. (1962) *J. Assoc. Off. Anal. Chem.* 45, 169-173).

content as compared with results calculated against the anhydrous standard. Such errors may be termed incorrigible after Crumpler and Yoe (ref. 1, p. 8; 5) because only the analyst can know of their presence and either prevent or eliminate them before the analysis of the sample. Similarly, errors in the calibration system such as an incorrect assumption of linearity over the concentration range of interest, or an improper calculation procedure (6) can lead to incorrigibility of the results.

Another incorrigible error which must be absent in an analytical determination is the presence of a direct interferent from the sample matrix. Although Wilson (3) has discussed interferents and presented a definition, the author prefers the following definition of systematic bias from an interferent: Any substance in the sample matrix which causes a response relative to the sample size which is either larger or smaller than that due only to the analyte resulting in an overall systematic error of an unacceptable magnitude. Common examples of interference bias are 1) the nonresolution of the analyte peak from the interferent in a chromatographic method, and 2) the same functional group reaction exhibited by the interferent as by the analyte.

2.2 Corrigible Errors

Constant and proportional errors are corrigible errors because the data taken during the analysis of an actual sample can be used to calculate their magnitude and appropriate corrections can be made. To deal with these corrigible errors, it is necessary to define them. A constant error is due to a significant relative response, positive or negative, not attributable to the analyte, which can either be measured directly or is mathematically related to a zero sample size arising from interferents in the matrix, or to a physicochemical property of the measurement system, and which is independent of the size of the sample. A proportional error results from a significant relative change in the analyte response per unit of analyte concentration ($\Delta R/\Delta C$), positive or negative, attributable to a parameter of the measurement system, procedure, or method, the magnitude of which is constant at all levels of the analyte concentration of interest. A significant relative response or relative response change for the case of constant error and proportional error, respectively, is one whose magnitude as a fraction of the nominal value would be unacceptable. Examples of constant error are the negative bias due to solubility product loss in gravimetry and the positive bias due to extraneous absorption in spectrophotometry. An example of a proportional error is recovery loss due to an inefficient extraction procedure. These corrigible errors are shown graphically in Figure 1.

2.2.1 Constant Errors

(*a*) *Determination of Constant Errors.*—The usual direct measurement of the constant errors in the measurement sample is familiar to analytical chemists as the blank. Two types of blanks are a necessary part of a response measurement system, namely, the instrument blank exemplified by the optical reference in spectrophotometry and the signal blank exemplified by the noise in a response tracing from a chromatogram or spectrogram. A chemical blank is the response obtained on a sample-free solution processed through the method, and the method blank or placebo blank is the response on an analyte-free sample processed through the method. The chemical blank is valuable as a trouble-shooting diagnostic device for tracking impurities from reagents, etc., which may be causing unacceptable levels of the method blank. The method blank is recognizable then as the quantity desired relating to the constant error correction term in analytical calculations.

A directly determined blank usually has several critically limiting deficiencies: Either there is no matrix present, as in the chemical blank, or there is no analyte present, as in the method blank. From the definition of constant error given above, it is evident that the blank as a measure of the constant error must be determined when both analyte and matrix are present. This seeming dilemma was uniquely resolved independently and coincidentally in the same year, 1947, by Kimball and Tufts (7) and Youden (8).

In titrating fluoride with thorium nitrate, Kimball and Tufts observed varying titer values (mg F/mL titrant) which were dependent on the amount of fluoride taken for titration. Their data have been reconstructed by the writer and are shown in Table 1. The titer has been calculated as the reciprocal of that used in titrimetric practice, mL titrant/mL F for representation according to the present conventions for standard response curves, as shown in Figure 2b. Kimball and Tufts recognized that the varying titer values were due to the presence of a constant error and that the zero blank value actually measured in the absence of fluoride was not the true value so they devised an ingenious algebraic procedure for calculation of the true blank. They titrated 2 levels of fluoride, 5 mg and 1 mg, and reasoned that each titration value contained the blank value. Subtraction of the lesser from the greater cancelled the blank value and division by 4 gave a titration value, blank-free equivalent to 1 mg fluoride which when subtracted from the actual measured value for 1 mg F yielded the desired blank; or, using the values in Table 1, B − (A −

Table 1. Kimball and Tufts procedure for blank determination

mg F, *X*	mL titrant, *Y*	Reciprocal titer value: mL/mg	Reciprocal titer value: mL/mg,[a] corr.
0.5	1.87	3.74	3.46
1.0	3.60 (B)[b]	3.60	3.46
3.0	10.52	3.51	3.46
5.0	17.44 (A)	3.49	3.46
7.0	24.36	3.48	3.46

[a] mL/mg, corr. = (mL − 0.14)/mg F.
[b] See text for explanation.

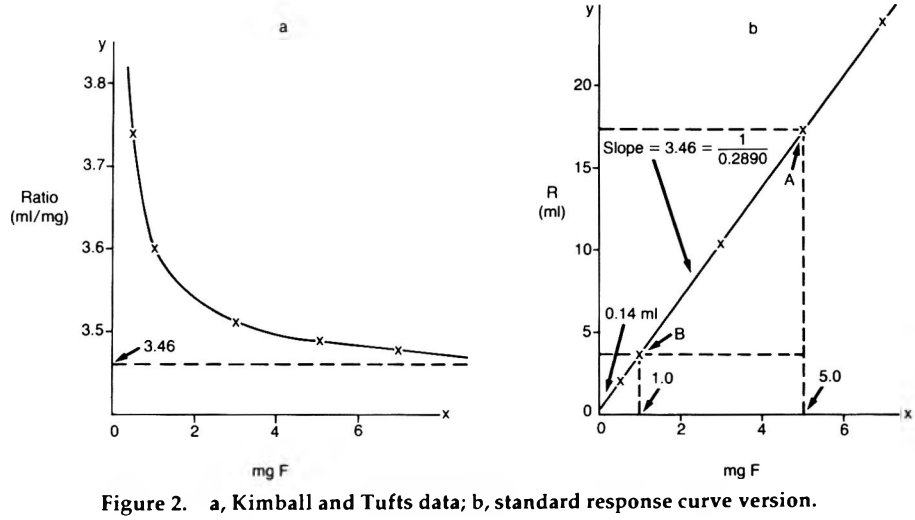

Figure 2. a, Kimball and Tufts data; b, standard response curve version.

Table 2. Effect of constant error: model data—full range

Range %	mg sample (X)	R (Y)	% S (Y')[a]	% S (Y″)	% S (Y‴)
—	6.803	0.252	24.90	13.13	1.37
—	13.34	0.316	19.10	13.10	7.10
—	40.00	0.579	15.13	13.13	11.13
—	66.64	0.841	14.33	13.13	11.93
—	93.33	1.104	13.99	13.13	12.27
75	119.2	1.358	13.79	13.12	12.45
100	158.9	1.749	13.63	13.12	12.62
125	198.6	2.140	13.53	13.13	12.72
				$\bar{X}$ = 13.12	
				RSD = 0.081%	

[a] Y', no blank corr.; Y″, YB corr.; Y‴, high blank, 0.120.
Youden regression
m = 0.009842 YB = TYB − SB
b = 0.185 = 0.185 − 0.125
% var. = 0.03 = 0.060
Std curve: $y = 0.075x + 0.125$

B)/4 = 0.14 mL. The true titer, then for the 5 mg F titration is 5/(A − 0.14) = 0.2890 mg F/mL titrant. The relative error resulting from a constant error is shown in Figure 2a where the correct reciprocal value of 3.46 mL/mg is approached asymptotically at the high end of the concentration range.

Although it is not the conventional treatment of titrimetry data, if the Kimball and Tufts data (Table 1) are plotted as shown in Figure 2b, a normal standard response curve is generated and by a linear regression, an intercept is obtained which is the same as the Kimball and Tufts blank and a slope which is their corrected reciprocal titer value. Generally, then, the intercept of a standard response curve over the linear dynamic range of interest is the system blank, the true value of the blank at zero analyte concentration.

As discussed by the author (4), the technique reported by Youden (8) in 1947 uniquely determined constant error in the presence of the sample matrix at zero sample concentration. The intercept of the Youden regression line from the variable sample weight/response curve (ref. 4, Figure 6) is the total Youden blank (TYB): TYB = YB + SB where YB is the Youden blank, the bias contribution from any analyte-matrix interaction, and SB is the system blank described above. The TYB value may be statistically compared to a directly determined blank (9) for reasons of analytical inquiry, but the only value to be used for corrective calculations is the TYB value because the Youden regression line is the standard curve of the analyte in the presence of the matrix and TYB is a mathematical parameter of that line functionality.

(*b*) *Effect of Constant Errors.*—(*1*) *In Calibration-Curve-Based Analyses.* The effect of a constant error in the determination of a titer value has been shown in Figure 2a, and in apparently the first recognition of the Youden regression technique by Linnig et al. (9), the same type relative bias error was noted. In a titrimetric method for the determination of fatty acid in solutions of synthetic rubber, values found decreased asymptotically as the amount of material to be titrated increased. This hyperbolic function, $Y = k/X$, is easily recognized when the sample size is varied over a broad portion of the dynamic range, but over a short range, particularly at the high end of the range, the observation can be misinterpreted.

To demonstrate the effect, the model data representing calibration curve-based analyses in Table 2 were prepared in which the sample size was varied over the nominal concentration range of 0 to 125% for the situations of the correct blank correction, undercorrection, and overcorrection. The standard response curve over the same analyte concentration range was linear and the slope/intercept straight line function was as shown in Table 2. The data are plotted in Figure 3. Both Linnig et al. (9) and Kimball and Tufts (7) in similar situations recognized the asymptotic characteristic of the plot as due to the presence of a constant error but if only the high concentration range of 75 to 125% were available for examination as is normally the situation in most analytical practice (4), the trend in the data, from high to low or low to high, either one being separately considered, would appear to be within the variance of the usual and acceptable 1 to 2% RSD method performance. Not infrequently,

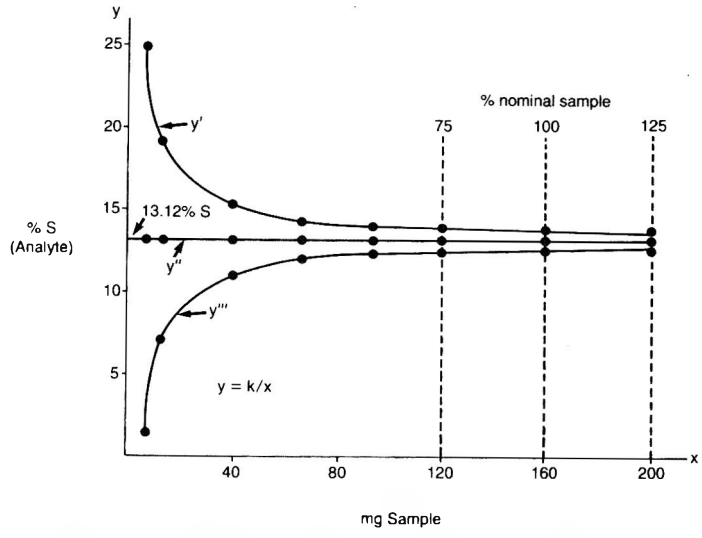

Figure 3. Effect of constant error: model data – full range.

the apparent trend, although possibly acceptable, would appear to be due to the presence of a proportional error, and a renewed investigation for a better extraction efficiency would be instigated as one possible method development option.

(2) *In Method of Standard Additions.* The presence of constant errors in the response values measured in the method of standard additions may be demonstrated as in Section 2.2.1(b). A general discussion of the method of standard additions has been presented in Part I (4). For this purpose, the responses measured on samples to obtain the Youden regression line and the method of standard additions line as they relate to each other are depicted in Figure 4a and b. The observation is clearly made that the response of the unspiked sample, A, is obtained under exactly the same analysis conditions according to the method description as were the data points obtained for the Youden regression curve. (In fact, sample A can be and usually is one of the Youden regression data points at the 40 to 50% level of the nominal concentration.) The point has already been made that these responses contain the bias contributions of the Youden blank (YB) and the system blank (SB). Their measure is the total Youden blank, TYB, and its contribution to the total unspiked sample response, A, is shown in Figure 4b. In the discussions in Part I it is brought out that this total Youden blank correction is not called for in the published literature, insofar as this writer is aware, and that only a few workers have called for a system blank correction. After algebraic subtraction of the TYB, the corrected analyte response, a, is used in the method of standard additions calculation as in the conventional practice (Figure 4c). [Compare with Figure 3 in Part I (4).]

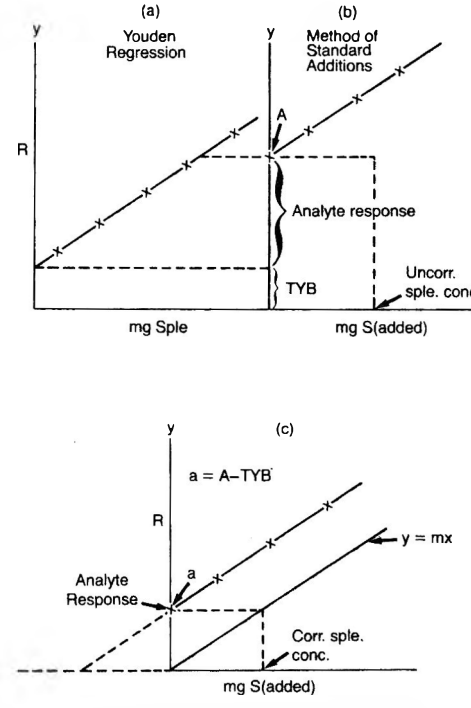

Figure 4. Total Youden blank (TYB) contribution to analyte respone in method of standard additions. See text for detailed description.

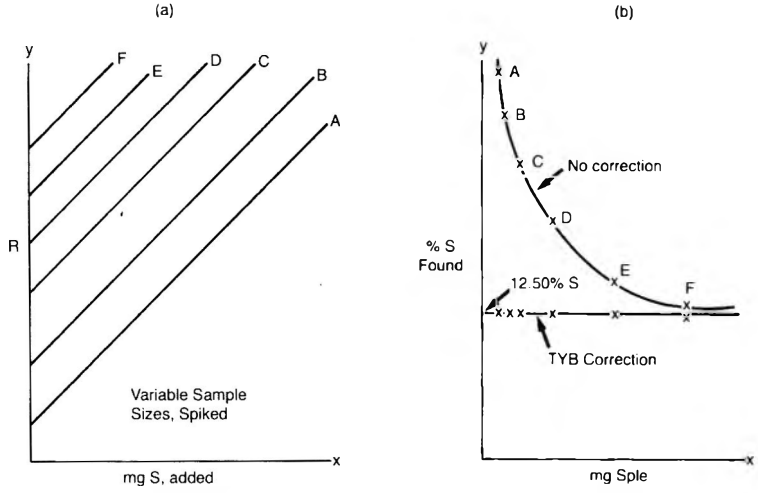

Figure 5. Relative error effect due to constant error in method of standard additions: model data.

Analogous to the preceding demonstration on calibration curve-based analyses, for the effect of noncorrection of the constant error in method of standard additions data, model data obtained by spiking samples of variable size resulting in a series of parallel method of standard additions curves are shown in Figure 5a, and the corrected and uncorrected calculated analysis data are plotted in Figure 5b. As before, the relative error effect is evident with the error largest at the lowest sample response level according to an apparent $Y = k/X$ function.

2.2.2 Proportional Errors

Determination of Proportional Errors.—Method of Standard Additions. The method of standard additions (MOSA) possesses an intrinsic property which is not readily discerned from a study of the literature, namely that the standard additions calibration line is an in situ normalization procedure for the proportional error of the method from the point of the procedure where the analyte spike is introduced. Normalization of the proportional error results in a change of the slope of the standard additions response curve from that of the standard response curve. Both the standard curve and the standard additions curve are responses of the reference standard, the former in a solvent system and the latter in the same solvent system but with the sample matrix present. Normalization occurs because the same procedural operation is performed on the unspiked and the spiked samples, so that a constant (that is, reproducible) percentage bias is introduced on the sample across the dynamic range.

Henning and Jackson (10), in comparing the slopes of the standard response curve and the MOSA curve, noted that they were parallel and that no interferences were therefore indicated in the method of additions system. Massart et al. (11) make a direct reference to the MOSA slope line which they term the recovery slope. The method of standard additions itself they term the recovery experiment which can "serve to evaluate the occurrence of proportional systematic errors." Unfortunately, Massart et al. only call for a statistical significance comparison of the slope of the recovery experiment with that of the calibration line obtained with pure standards, or alternatively, to make a statistical significance comparison of the results from standard additions with those obtained by using the direct determination. The use of the ratio of the standard additions curve slope to that of the standard response curve slope as the calculation of the recovery from the method of standard additions data was reported by the author in 1978 (12) and has since been used for that purpose but that this recovery factor was in fact the proportional error of the system was also not then realized. In the technique now proposed, the standard curve results from the analysis after correction for the constant error, the Y" values of the model data in Table 2 are divided by the proportional error factor (PEF), 1.05 (Figure 6), and the correct result of 12.5% S (analyte) in the sample is then obtained.

3.0 Functionality of the Youden Regression and the Method of Standard Additions

According to the literature (4), the method of standard additions has always been treated and

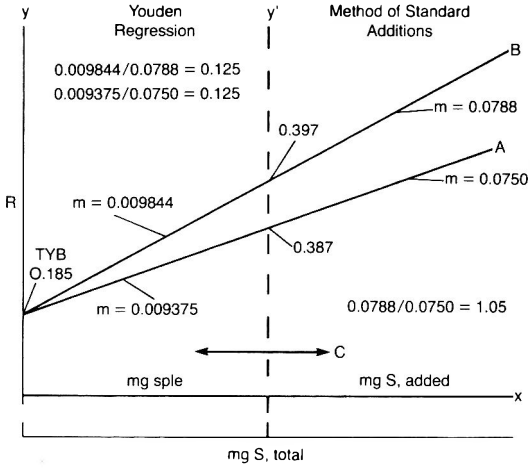

Figure 6. Linear functionality of Youden regression and method of standard additions. Standard curve in the matrix: model data.

Std curve $y = 0.0750x + 0.125$

Sample 12.5% S

line A: YB 0.060R units; zero PE

line B: YB 0.060R units; 5% PE

used as a free-standing technique with its main power in the characteristic that analyses can be performed without using a standard curve. It is generally understood that the method of standard additions technique generates its own calibration curve and that this calibration curve is in the presence of the matrix, in contrast with the standard curve. The functionality of the method of standard additions curve is expressed as $Y = mX + a$, where a is the intercept parameter and is expressly the response of the unspiked sample. These considerations are discussed in Part I (4). Although of great practical importance to applied analysis, this independent functionality is incidental to the true functionality of the method of standard additions line as shown in Figure 6.

The overall plot in Figure 6 is that of a standard curve, response R of analyte vs the concentration, mg *S* (analyte). This total analyte concentration range however is composed of 2 parts, the left side the by now familiar Youden regression plot and the right side, the conventional construction of the method of standard additions plot. In the Youden regression plot, the mg sample abscissa scale is actually an analyte scale since mg sample × %S/100 is equal to mg *S* so that the Youden regression is actually on the same overall mg *S* scale. In the method of standard additions, the selection of a sample size merely picks a point on the abscissa scale as shown by the slider C with its resultant value of the MOSA intercept. The analyte spike additions merely extend the standard curve range.

Thus, it is obvious from Figure 6 that the true functionality is $Y = mX + TYB$. This is why the TYB value must be algebraically subtracted from the unspiked sample response when rendering the MOSA technique (Figure 4). The overall curve is a standard curve in the matrix with a constant slope equal to the MOSA portion of the curve since this portion is directly in the analyte concentration unit. Since the Youden regression slope value contains the concentration term of the sample, the ratio of the Youden regression plot to that of the MOSA is directly the percentage assay of the sample if both plots have the same abscissa scale units. If not, a scale factor (SF) adjustment is required. Generally, (Youden regression slope) (scale factor)/MOSA slope = dec. % assay. In Figure 6, this ratio is 0.125 or 12.5%.

In Figure 6, line B was built-in with a 5% proportional error (PE), whereas line A had no PE, and it can be noted that the slope of line A is the same as that of the standard curve. In the preceding section, the change in slope ratio of the MOSA curve to that of the standard curve is a direct measure of the proportional error, and in Figure 6 this ratio is 1.05. Also, from Figure 6, it is evident that in the presence of a proportional error source, the change in the slope of the MOSA curve (line B) is also accompanied by a correlatable functional change in the MOSA intercept (compare with the line A MOSA intercept), an observation or a conclusion that has not been hitherto possible from a study of the literature, or intuitively from experience. This change of the intercept with the change in the slope of the MOSA curve is not attributable to the negative correlation effect discussed by Mandel (13) but is a change concomitant with the effect. (See Part I and Figure 7, Part I for a discussion of the negative correlation effect.)

4.0 Experimental

Following the pattern of the model data, the 3 components comprising the experimental data are: 1) the standard response curve, 2) the Youden regression curve, and 3) the method of standard additions curve. For the first application, the analysis of bethanechol in bethanechol tablets was selected because an automated air-segmented continuous flow colorimetric method

Table 3. Analysis of bethanechol tablets (%) by 3 different methods: corrected and uncorrected calculations

	Day 1					
	Conventional			Proposed		
Parameter	CFA	Grav.	Kjel.	CFA	Grav.	Kjel.
Std curve assay[a]	97.6	110.2	108.2	101.0	102.3	101.0
RSD, %	8.6	2.58	5.31	2.0	3.6	2.5
% Var.	—	—	—	2.2	2.5	3.0
% Var., std curv	0.7	3.5	1.3	0.7	3.5	1.3
% CE[b]	—	—	—	−5.9	1.1	4.7
PE[c]	—	—	—	105.0	106.4	101.5
MOSA assay[d]	89.6	106.7	102.9	102.2	101.5	101.3
Slope ratio assay	—	—	—	100.8	102.3	100.0

[a] Average of 6 determinations from Youden regression samples.
[b] % CE = $(YB/\overline{Y})(100)$.
[c] % PE = (slope MOSA/slope std curve)(100).
[d] ** Average of 2 MOSA spiked sample levels.

had been developed from a manual method and in addition, there were 2 other existing USP methods, a tetraphenylboron gravimetric method and a digestion-titrimetric (Kjeldahl) method. Each method was carried out on separate days using the same carefully prepared composite pool of production tablets. In each case, the standard response curve was run over the entire linear dynamic range of the method using 6 data points. The Youden regression curve was generated by varying the sample weight so that responses over the same linear dynamic range were obtained, again 6 data points. For the MOSA curve, sample weight sizes at 50 and 75% of the nominal concentration range were selected for the spike additions of 3 increments in each case so that the highest level of response was 150% of nominal and remained within the linear dynamic range of both the standard response curve and Youden regression curve. Two spike levels were chosen to arrive at a better average value for the slope of the MOSA curve and in the initial work to demonstrate that the slope of the MOSA curves at the 2 levels were statistically the same. All data points for any one method rendition were taken as a set, that is, in the same day with no procedural variations, changes, or conscious adjustments of any kind during the set. The data are shown in Table 3.

To demonstrate the reproducibility of the proposed calculation technique, the CFA set was repeated on the same sample 2 weeks later with the results shown in Table 4.

Another application to one of our cough and cold tablets which contains 3 active drugs is shown in Table 5. The method uses the newer HPLC multiple analyte technique and was one of several then under study. The data were taken as part of the alternative method development program for this product.

4.1 Discussion of Experimental Results

The overall effectiveness of the calculated correction is evident from the data in Tables 3, 4, and 5. In all the tables, the 3 assay values, stan-

Table 4. Reproducibility of CFA analysis of bethanechol tablets (%) after 2 weeks

	Conventional		Proposed	
Parameter	Day 1	Day 2	Day 1	Day 2
Std curve assay[a]	97.6	109.7	101.0	103.2
RSD, %	8.6	6.7	2.0	2.4
% Var.	—	—	2.2	2.6
% Var., std curve	0.7	4.4	0.7	4.4
% CE	—	—	−5.9	10.9
% PE	—	—	105.0	95.5
MOSA assay	89.6	128.3	102.2	102.3
Slope ratio assay	—	—	100.8	102.0

[a] See footnotes *a–d*, Table 3.

Table 5. Simultaneous multiple analyte determination of 3 active ingredients in cough and cold tablets: corrected and uncorrected calculations

Parameter	Conventional			Proposed		
	CPM	PEH	PTC	CPM	PEH	PTC
Std curve assay[a]	9.44	50.36	128.2	9.39	49.88	133.6
RSD, %	4.1	2.7	1.9	1.7	1.2	2.3
% Var.	—	—	—	2.5	1.2	1.8
% Var., std curve	2.0	0.6	1.5	2.0	0.6	1.5
% CE	—	—	—	−3.9	−1.6	−0.6
% PE	—	—	—	106.0	103.6	96.9
MOSA assay	7.92	51.34	128.8	9.22	49.90	132.6
Slope ratio assay	—	—	—	9.38	49.95	133.0

[a] See footnotes *a–d*, Table 3.

dard curve, MOSA, and slope ratio, which the experimental data set provides, are in agreement with one another within the variance of the methods. It should be recognized that if the slope ratio assay results disagreed unacceptably with the other 2 calculation results, this finding would be an important diagnostic indicator that the entire set is suspect, and the experimental design should be re-examined, and possibly the method itself.

In Table 3 the expected finding that the constant and proportional errors in each method are different is shown, but not so expected were the results in Table 4 which revealed their striking difference from set to set. System stability must be good enough so that the same experimental conditions apply to all samples within the set. How different they are from set to set under experimental conditions more akin to a repeatability situation is yet to be determined if the technique is to have possible application to routine analysis.

Certain of the assay results in Table 5 deserve comment; for example, the uncorrected PTC values from the standard curve and MOSA techniques agree very well, yet they are significantly different from the corrected assay results. Also, the CPM standard curve uncorrected and corrected assay results agree very well but the CPM standard curve uncorrected assay result differs significantly from the uncorrected MOSA result.

The situations noted are the effects of the positive and negative constant and proportional bias errors, resulting in fortuitous cancellation and apparent agreement. Thus, the uncorrected values of 128.2 and 128.8 for PTC would be completely acceptable if no other data existed, but are in fact a significant 3.5% lower than the correct value of 133.0, the overall $\overline{X}$ value from the corrected assay results. Again, the uncorrected value of 9.44 and the corrected value of 9.39 for CPM is due to a cancellation of error, in this case resulting in an even more fortuitous correct result. However, the behavior of the errors is highlighted by the uncorrected MOSA result where only the CE is operative. This yields an unacceptably low 7.92 assay result, an example of the commonly observed non-agreement of standard curve and MOSA results. Cancellation of errors is bad analytical practice because of their imposition of an arbitrary and restricted linear dynamic range, and these correctional data exemplify their effects.

Estimates of the precision of the method from the data set can be obtained in several ways. The first is obtained by examining the % variation of the standard response curve as obtained from regression analysis of the standard curve and defined as the (std error est./$\overline{Y}$) (100). In Table 5 the value for CPM is 2.0%. The second estimate is the % variation of the Youden regression curve, from Table 5 for CPM, 2.5%. Most often, the % variation from the Youden regression data is higher than that from the standard curve because the former entails the entire procedural variation on the sample whereas the latter is only the final measurement portion of the procedure. In simple cases, however, such as in a dilute-and-inject method where sample preparation steps are simple, both values may be equally weighted estimates of the sample method procedure variation. The third estimate is the RSD of the sample data set from the Youden regression determinations which have been calculated, corrected or not for the Youden blank constant bias error by interpolation using the standard response curve. This estimate of the precision of the method, the RSD of the sample data set (from Table 5, for CPM, 1.7%) embodies the entire

Table 6. Youden regression sample analysis data and standard curve calculations for CPM in Table 5

Level	Sample, g (X)	PH (Y)	PH (corr.) Y'	mg CPM/g[a]	mg CPM/g[a] corr.	mg CPM/g corr. for PE
25	0.0968	1814	2145	8.76	9.98	9.42
50	0.1891	4412	4743	9.42	10.05	9.49
75	0.2859	7026	7357	9.52	9.93	9.38
100	0.3813	9368	9699	9.35	9.66	9.12
125	0.4775	12608	12939	9.90	10.15	9.58
150	0.5734	14914	15245	9.69	9.90	9.35
			$\overline{Y}$	9.44	9.95	9.39
PH (corr.) = PH − YB			RSD, %	4.1	1.7	1.7
					% Var.	2.5

[a] mg CPM/g = [mg CPM/mL (std curve)](200)/g sample, from Y or Y' value, respectively.

procedural operation, the sample variation, and the standard curve variation. Therefore, this value should reported in the method description statement, whereas the first and second estimates are useful diagnostics.

4.2 Illustrative Calculations

As an example of the stepwise calculations, the data reported in Table 5 for CPM shown in the block are used:

1). Following the method description, obtain the standard response, Youden regression, and MOSA curve data. The Youden regression sample data are shown in Table 6 and the MOSA curve data are shown in Table 7.

2). Using a linear regression analysis technique, obtain the respective slope/intercept straight line mathematical functions. Examine the plots (manual or plotter) visually for linearity. (See step 8 for another test of linearity.) In the case of the standard response curve and Youden regression curve, drop any datum points at either the low and/or high end of the concentration range that do not fall on the linear portion of the function. All use of the linear dynamic range must thereafter adhere to the defined linear dynamic range. If the MOSA plot is not linear, the MOSA technique is *not* applicable and any calculations using MOSA data *cannot* be made. (Non-linear MOSA applications [see Part I (4)] have not been studied.)

Table 7. Method of standard additions: analysis data for CPM

	Sample/spike level	
	0.190 g/50%	0.285 g/75%
mg CPM/mL (added) X	PH Y	PH Y'
0	4412 (4210)[a]	7026 (6981)[a]
0.005	6976	9822
0.010	10107	12796
0.015	13161	15730
0.020	16243	—

[a] Intercept values from linear regression analysis.

3). Report the respective regression data:

a). Standard response curve

Y (PH) = 556 232X (mg CPM/mL) − 543

Rel. Y-intercept = −5.8%

% var. = 2.0

b). Youden regression curve

Y (PH) = 5528X (mg sample/mL) − 874

Rel. Y-intercept = −10.5%

% var. = 2.5

$\overline{Y}$ = 8357

c). Method of standard additions curve

50% level: Y (PH) = 596 940X (mg CPM/mL) + 4210

Rel. Y-intercept = 41.4%

% var. = 1.8

75% level: Y (PH) = 581 720X (mg CPM/mL) + 6981

Rel. Y-intercept = 61.5%

% var. = 0.5

Average MOSA slope = 589 330 PH mL mg^{-1}

Note: Relative Y-intercept values for the MOSA curve should be no less than about 40% of the nominal concentration response so that a significant portion of the unspiked sample response is due to the analyte, but at the same time a total response for the last increment should be no less than 2 times the unspiked sample response.

4). a). Calculate the Youden blank and % constant error

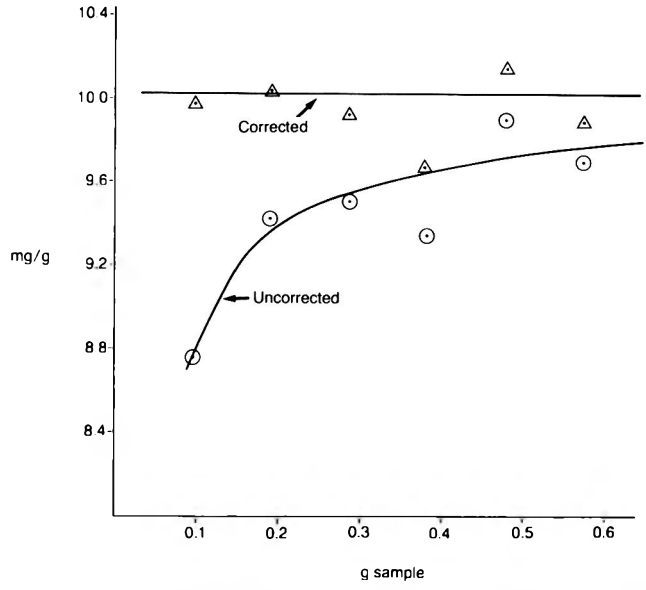

Figure 7. Assay values as function of sample size – effect of constant error. Data from Table 6.

YB = TYB − SB
= −874 − (−543)
= −331

% CE = (YB/$\overline{Y}$)(100)
= (−331/8357)(100)
= −3.9

b). Calculate % proportional error

% PE = (slope MOSA curve/slope std curve)(100)
= (589 330/556 232)(100)
= 106.0

5). Calculate the slope ratio assay

mg/g CPM = [(slope Youden regression curve)(SF)/slope MOSA curve]

$$\frac{5528 \text{ mL (mg}^{-1}\text{ sample)}(1000)}{589\,330 \text{ mL (mg}^{-1}\text{ CPM)}}$$

= 9.38 mg CPM/g sample

Scale factor of 1000 to convert mg CPM/mg sample to mg/g.

6). Calculate corrected assay values by interpolation of the Youden regression sample data using the standard response curve (see Table 6):

a). Algebraically subtract the YB value from the observed PH (Y) values to obtain the PH (Y') corrected values, 1814 − (−331) = 2145.

b). From the standard curve equation, transformed, calculate

mg CPM/mL, corr.: $X = (PH_{corr.} + 543)/556\,232$

$X = (2145 + 543)/556\,232$
= 0.004 833

c). Calculate mg CPM/g, corr., from the expression:

mg CPM/g, corr. = [(mg CPM/mL)(diln factor)]/g sample
where diln fact = 200

mg CPM/g, corr. = (0.004 833)(200)/0.0968
= 9.98

7). a). Divide the mg CPM/g, corr., value by the PE factor to obtain the final corrected assay value:

mg CPM/g, corr., total = (mg CPM/g, corr.)/PE factor
= 9.98/1.06
= 9.42

(b) Calculate $\overline{X}$ and RSD of the corrected group:

$\overline{X}$ = 9.39 mg CPM/g
RSD = 1.7%

8). Plot the mg CPM/g values, corrected and uncorrected vs g sample and obtain the linear analysis regression data. See Figure 7. The slope of the corrected data should be zero. From the regression data, examine the 95% confidence intervals and ascertain whether the value of zero is included in the respective intervals. For the example, CI for the corrected data does contain zero and for the uncorrected data, does not.

9). Calculate the MOSA assay value by correcting the MOSA regression intercept value, A (Figure 4b). Subtract the TYB value, −874, to obtain the corrected MOSA regression intercept value, a (Figure 4c). Substitute the value of a for Y in the transposed form of the expression of the

MOSA standard curve, $X = Y/m$.

50% Level

$X = [4210 - (-874)]/589\,330$

$= 0.008\,627$ mg CPM/mL

mg CPM/g = (0.008 627)(200)/0.190

= 9.08

75% Level

$X = [6981 - (-874)]/589\,330$

$= 0.01\,333$ mg CPM/mL

mg CPM/g = (0.001 333)(200)/0.285 g

= 9.35

Av. CPM/g = 9.22

5.0 Summary and Conclusions

The calculation technique presented serves both as a diagnostic for the quantitation of constant and proportional errors and as a procedure for using the information to calculate a correct analytical result on the sample during the course of the analysis regardless of the presence of errors. With the new technique, results obtained from the standard response curve agree with the results obtained from the method of standard additions within the variance of the random error of the method. A new insight of the fundamental relationship between the Youden regression and the method of standard additions permits an assay of the sample to be obtained from the simple ratio of their respective slopes.

Obviously, the magnitude of the relative error in analysis results due to an incorrect constant error correction, or a noncorrection, is much higher for samples with low levels of analyte, and particularly so in trace analysis. Perhaps the observation by Horwitz (14) of the 2-fold decrease in precision for each 100-fold decrease in analyte concentration might be due to this relative error effect at these low analyte concentrations as suggested by examining Figure 3.

As long as incorrigible bias errors are absent, the correction for the corrigible bias errors leads to a correct analytical result by a method, and hence, different methods will also produce the same analytical result on the same sample. If the results by different methods do not agree, an indication as to the presence of incorrigible bias errors is made provided only that the system has been shown to be stable enough to ensure constancy of the corrigible errors during the time required for the determination of the data set.

The data obtained by the new technique provide all that is necessary to meet the GMP/GLP requirements for method validation performance characteristics of linearity (i.e., sensitivity) and accuracy (i.e., bias and precision) in a fraction of the time required by the conventional approaches (4) because a minimum of only 13 data points are needed. Problem out-of-specification samples in quality assurance which raise the question of which is out of control, the process or the method, can now be addressed by the simple expedient of producing a correct result. Sample analysis by the method of standard additions requires only the TYB value and no standard curve data at all so that application of the technique to QA routine samples is possible. Further studies concerning these and other applications are in process.

Acknowledgment

I thank my director, Louis R. Lieto, for his encouragement and valued consultations. My colleagues, Jay G. Lehman, Philip J. Palermo, and Lal Kamra, helped acquire the data and provide the foil so vital to the development of the concepts.

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Today's Chemical Realities

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The difficulty with current food safety laws arises from the apparent inability of the toxicologist to determine the biological significance of the extremely low concentrations that the chemist can currently measure. In discussions of this technical ability, however, the practical limitations of chemical measurement science are rarely mentioned. Some of these limitations are: (1) The reliability of identification and quantitation decreases exponentially as concentration decreases; (2) at very low concentrations baseline effects become the limiting factor; and (3) the cost of the analysis becomes exorbitant. The practical reality of trace chemical analysis in the interlaboratory environment must answer the question: When does the cost of the analysis exceed the value of the information gained as to the identity and amount of the material present, considering also the uncertainties associated with the analysis itself and with the biological effect of that concentration? The current practical limit of measurement is in the low parts per billion, e.g., aflatoxin contamination. 2,3,7,8-Tetrachlorodibenzodioxin can be determined in fish at the moderate parts per trillion level, but at an estimated cost in salaries and expenses of roughly $1000 per individual value reported, without capitalizing our $2,000,000 mass spectrometry laboratory.

About 50 years ago, G. E. F. Lundell of the National Bureau of Standards published his classic paper (1), "The Chemical Analysis of Things as They Are", in which he discussed 4 major analytical factors: the analytical sample, the method of analysis, the accuracy of the result, and the cost of the analysis. Lundell chose his topic "because so many talks and articles on analytical subjects deal with 'The Chemical Analysis of Things as They Are Not'." He pointed out that a system containing 10 to 20 diverse components cannot be handled on a strictly scientific basis. Today's chemical realities require that we deal with systems such as formulated foods, waste waters, and body fluids, which contain thousands of distinct chemical entities, but we are still concerned with those same 4 factors.

Until the passage of the Pesticide Chemicals Amendment to the Federal Food, Drug, and Cosmetic Act, the Food and Drug Administration was concerned with substantial residues of poisonous and deleterious substances that could be measured with a fair degree of confidence. Some of the informal tolerances that were enforced included arsenic at 3.5 ppm and lead and DDT at 7 ppm. The methods of analysis used to enforce these informal tolerances are now considered obsolete: the reading of a stained paper strip in the Gutzeit method for arsenic; the colorimetric and later spectrophotometric methods for lead by dithizone and DDT by the Schechter-Haller reaction. Use of these methods took a good deal of skill, and the reliability of the analytical data they produced varied considerably. The arsenic method included a relatively specific separation step which made the method rather rugged. In contrast, the methods for lead and DDT could be subject to considerable interference from other metals and from other new organochlorine pesticides, respectively. At that time, trace quantities of pesticides (about 0.1 ppm) could not be measured with any degree of confidence and therefore were considered without chemical or biological significance.

In 1954, Congress passed the Pesticide Chemicals Amendment to the Federal Food, Drug, and Cosmetic Act. This amendment made the petitioner prove, before marketing a toxic material, that it could be used safely. Previously it was up to the Food and Drug Administration after the material was in use to prove that it left harmful residues. The amendment also authorized the establishment of formal tolerances to protect public health and it empowered the Secretary (now the Administrator of the Environmental Protection Agency) to establish the residue tolerance "at zero level if the scientific data before the Secretary do not justify the establishment of a greater level." The interpretive regulations (originally 21CFR120, now 40CFR180) defined the zero tolerance as meaning that "no amount of the pesticide chemical may remain on the raw agricultural commodity when it is offered for shipment." This definition was obviously directed toward defining the point of examination, rather than as a definition of "zero." The zero tolerance level was intended to be a catchall to avoid residues from incompletely tested chemicals, extremely toxic chem-

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icals, and rapidly and spontaneously degradable or removable pesticides, as shown by the examples of reasons given in the regulations for formally establishing a zero tolerance.

In 1958, the Food Additives Amendment was added to the Federal Food, Drug, and Cosmetic Act. It contained the Delaney clause, which prohibited the addition of any carcinogenic chemical to food. However, the important analytical section for chemists was contained in a later modification of the Delaney clause, the so-called DES proviso, which exempted feed additives from the application of the prohibition if "no residue of the additive will be found (by methods of examination prescribed or approved by the Secretary. . .) in any edible portion of such animal after slaughter. . ."

Both the zero tolerance and the no residue provisions were enacted before the full effect of the instrumental revolution initiated by gas chromatography had been felt. After the introduction of the electron capture detector, which reduced the limit of determination for chlorinated compounds by 2 orders of magnitude, it became apparent that the law had unwittingly set up an intolerable contradiction. Chemical measurements are based on the presence of something, not on its absence. The "something" may be a charged particle, an aggregate of particles, or electromagnetic waves whose effects can be magnified in the measuring system. Most measuring systems require millions or billions of particles for activation. Below this activation level we have the equivalent of discontinuous chaos where probability considerations determine whether sufficient particles or waves occasionally agglomerate to trigger the measurement system. Even those detection systems which are said to respond to a single particle cannot escape this paradox. After the first particle has been measured, there is presumably nothing (in an absolute sense) left to verify that this particle was really the particle sought. Therefore, a second, a third, a fourth particle, and so on is needed until the analyst has achieved confidence in the repeatability of the measurement. In either case, whether we are dealing with a single particle or the millions necessary for a measurement, we have no way of knowing that the zero of the measuring instrument is a "less than" or an "absolute zero."

In addition to this theoretical limitation on the ability to determine a real absolute zero, there are 3 additional limitations in chemical analysis: (1) the experimental limitation from baseline effects—inherent physical, chemical, and electronic noise; (2) the empirical limitation arising from the decreasing precision of measurement systems as they approach zero; and (3) the practical limitations imposed by budgetary restraints.

Baseline Limitations

Let us assume an absolutely specific detection system—one that responds exclusively to the substance we desire to measure. Even such a selective detection system is not so specific that it is unresponsive to continuous extraneous sources of signals which may bracket the discontinuous signal source. In dealing with elements, all of our construction materials and sample and laboratory containers are contaminated with minute amounts of numerous common elements as impurities. These impurities in the aggregate contribute to a general background signal which contains energy identical to the authentic signal and thus is a limiting factor to our attaining a zero measurement.

In the case of organic molecules, electronic noise in the measuring system provides a limiting factor. All of the techniques for minimizing this inherent noise consume some of the wanted signal in the process, thus disqualifying such systems from attaining the zero area. Signal averaging, for example, through multiple scans of the energy area of interest, achieves a level of random noise ever closer to zero as it integrates a low-level real signal at a specific energy level location. But here the need for multiple scans requires a finite amount of the wanted material and the greater the confidence desired in the measurement, the larger the amount of material that must be available for the integration.

In addition to instrumental and laboratory baseline limitations, low background levels of numerous organic chemicals already exist in our environment of air, water, and soil from the manufacture, application, and disposal of thousands of consumer, agricultural, and industrial chemicals. Almost all animal tissues still contain at least parts per billion of DDT, although its use was discontinued a decade ago. Many common pesticides have been dispersed into the environment despite their very slight solubility in water and low vapor pressure (2). Settle and Patterson (3) have shown that human activity has resulted in an elevated lead concentration in many biological species. Donaldson (4) points out that almost 1300 volatile synthetic organic compounds have already been "found" in environmental waters at concentrations of about 10^{-8} (10 ppb) and above, and that as our ability to

measure lower concentrations increases, the number of compounds found increases accordingly. He states, "Based upon the number of compounds detected by current methods, one would expect to find every known compound at a concentration of 10^{-12} g/L [10^{-15} expressed as a fraction] or higher in a sample of treated drinking water."

The technical ability to measure very low levels of chemicals cannot be equated to biological significance. The toxicologist is unable to determine the significance of the extremely low levels of chemicals which the chemist is able to measure. The sheer practicalities of the logistics and expense of animal experiments (5), and the large number of organisms required to prove statistical significance of biological phenomena in the face of biological baselines and variability, preclude ever being able to use direct experimental evidence to interpret the existence of toxicological effects at low concentrations. Eventually, however, it may be possible to interpret biological effects in *in vitro* systems, where large numbers of cells can be used. Such systems may provide stable statistical parameters for biological effects that might be extrapolated through comparative biochemistry to effects on humans. However, these biological systems will be subject to the same theoretical limitation of determining "zero" as chemical systems.

Reliability Limitations

The reliability of chemical analysis is judged on the basis of how close actual assays are to the true value. Unfortunately, we rarely know the true value of any of the materials we analyze. In macroanalysis we sometimes are able to use fundamental physical and chemical principles to validate our measurements. But at trace (10^{-6}) and subtrace (10^{-9}) concentrations, the true value becomes increasingly difficult to determine. We can measure at low levels only with instruments. Unfortunately instruments, because of their very stability, can reproduce an erroneous value with high precision. If the prior chemistry is defective, the instrument is unaware of this fundamental defect.

Most instruments are devices in which a response due to a sample is compared with the equivalent response from a standard, usually the pure material serially diluted to the working concentration. There are a number of problems with this approach as well as with the alternative approach of standard additions. First, pure, stable reference compounds are often very difficult to obtain or maintain. Second, standards prepared from the pure reference material in solvent provide no information about the errors inherent in the various steps from weighing the analytical sample to presenting the final prepared, cleaned-up solution to the instrument. Third, standards added to the bulk of a material often do not behave in the same way as naturally and biologically deposited material, due to metabolic or environmental changes or equilibrium factors.

The realities of standards are illustrated in the results of the American Oil Chemists' Society Smalley Check Sample Program as reported by McKinney (6). In this program, samples of various seed meals are analyzed for aflatoxins by subscribers in order to judge for themselves their analytical proficiency. The average coefficient of variation (CV) of the results from 13 series over a 5-year period is approximately 55% at about the 40 ppb level. However, 6 independent solutions of pure aflatoxins at a concentration of about 1 ppm, which merely required spotting, developing, and comparison with the analyst's own standards, without extraction or cleanup, showed a between-laboratory CV of about 70%. This suggests that there are serious laboratory housekeeping problems with the preparation or maintenance of the participants' standards.

Not only do we have problems with the extent of our systematic errors, we also have problems with the reproducibility of our results as we go down the concentration scale. Recently we have been able to quantitate this effect by an examination of the cumulative results of AOAC collaborative studies (7). A summary of the interlaboratory variability expressed as the coefficient of variation (CV) as a function of concentration expressed in powers of 10 is given in Figure 1. This relation is now a summary of over 200 individual collaborative studies, each involving at least 6 laboratories, on analytes ranging from pure drugs and pesticides, through food nutrients, trace nutrients, drugs in feeds, pesticide and drug residues in biological tissues, trace elements, and aflatoxins. The remarkable aspect of this curve is that it appears to be independent of analyte, matrix, method of analysis, and measurement technique.

It is important to recognize that this curve represents interlaboratory precision, not intralaboratory precision. All analysts individually claim much better precision. In the trace analysis field, however, their performance must be judged relative to other laboratories, not relative to themselves. The Food, Drug, and Cosmetic Act, by requiring that a portion of the official

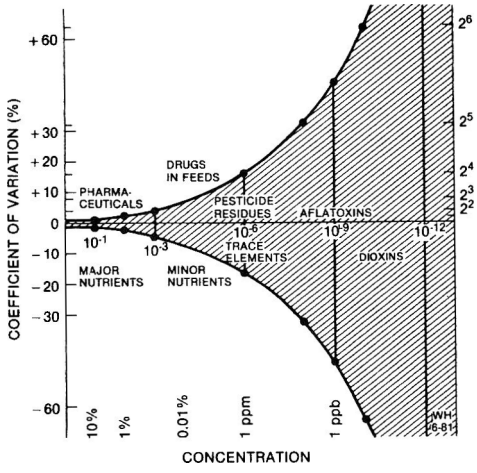

Figure 1. General curve relating interlaboratory precision to concentration. Coefficient of variation is plotted as % on left and as powers of 2 on right vertical axis; concentration is plotted as powers of 10 on horizontal center axis (7).

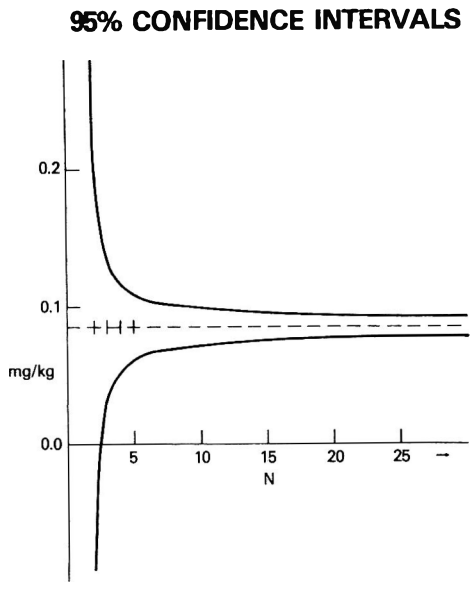

Figure 2. The 95% confidence intervals about a mean of 0.085 mg/kg (ppm) with a CV of 23% as function of the number of laboratories contributing to the mean.

sample be made available to the owner or shipper of the material sampled, makes it mandatory that the Food and Drug Administration (FDA) operate in an interlaboratory environment.

This curve indicates typical variability between laboratories, which in turn restricts the estimation of systematic error and the limit of reliable measurement. It restricts estimation of the systematic error because the constant bias can only be measured by means of methods which operate with the inherent random error exhibited by the curve. For example, FDA has proposed (8) that it will not accept methods which give average recoveries (systematic errors) outside the range of 60–110% at concentrations below 0.1 ppm (10^{-7} when expressed in powers of 10) when dealing with residues of drugs suspected to be carcinogenic. For simplicity, let us assume an acceptable 85% recovery (midway between the extremes) at 0.1 ppm, which, from the general CV/concentration curve of Figure 1, corresponds to a CV of 23%. Now let us calculate the confidence limits (CL) around the assumed mean of 0.085 mg/kg (ppm) from the statistical formula $CL = x \pm (ts/N)$ as a function of the number of laboratories (N) contributing to our data point. The result of this calculation, shown in Figure 2, is interpreted as follows: Assuming a normal distribution, and for simplicity assuming also that the intralaboratory variability is negligible (no variability), the true recovery of the N laboratories will fall within the 2 confidence limits 95% of the time. Such confidence interval bounds cannot predict where any actual average from, say, 5 laboratories will fall; it can only predict that the confidence interval will encompass the true answer 95% of the time.

Note that we assumed average recoveries from any given laboratory of 60–110% and took the midpoint of that range as our reference point for the recoveries from all laboratories. This is the actual mean recovery anticipated for the multiresidue pesticide method used by FDA as applicable to almost 200 pesticide residues and related materials in foods. The curve shows that a minimum of 5 laboratories (as suggested by Youden) is required for the CL curves to approximate the practical limits suggested by FDA for the results from a single laboratory. More laboratories will provide us with a more representative sample of laboratories and therefore a less variable standard deviation between laboratories. Fewer laboratories, however, will give us an almost uninterpretable wide confidence interval to work with. Note also that with only 2 laboratories, our lower confidence bound is negative, i.e., less than zero.

The curve of Figure 1 represents the smoothed average reproducibility of methods approved by the AOAC, which have been tested by laboratories under conditions that elicit their best performance. Such tests show the best operation of methods. Routine performance can be expected

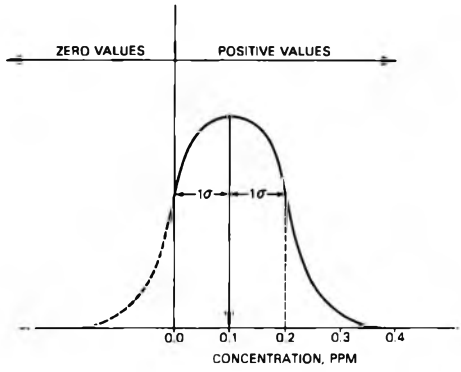

Figure 3. Normal distribution curve of a frequency distribution whose standard deviation is equal to its mean (CV = 100%). Note that one-sixth of the values will be below zero.

to be considerably poorer. For example, the AOAC aflatoxin methods in collaborative studies show an average CV of about 30% at levels above about 10 μg/kg (ppb). The results on the Smalley Series, where any method of analysis is permitted, show a variability of almost twice that value. Furthermore, we cannot fix our confidence interval for systematic error from a single laboratory or from a few laboratories with any degree of reliability because of the limitations imposed by the high CV. These statistical considerations are the basis for the statement by Currie and DeVoe (9) that to determine a systematic error of the same magnitude as the random error requires at least 15 observations.

The random error, as measured by the CV, also has a critical bearing on our ability to fix the limit of reliable measurement. Consider what happens to the frequency distribution of analytical results in a normal distribution when the standard deviation equals the mean, or CV = 100%. Figure 3 shows this situation, which would be expected to occur in trying to measure dioxins at the parts per trillion (10^{-12}) level; 17% of the values will be below zero and they would be reported as false negatives. As pointed out by Thompson and Howarth (10), they are legitimate values. The blank happens to be greater than the measurement. These estimates of false negatives are conservative because the blank has a frequency distribution whose values need not be distributed normally. If the distribution of values whose CV = 100% is skewed, and if the blank also has a frequency distribution, i.e., it is not always zero, there will always be negative values, although the level where this occurs may vary.

The presence of negative measurements in trace analysis is not ordinarily apparent for a number of reasons. The most important reason is that when a negative value appears in the course of ordinary work, it is usually equated to zero. Second, even in operating with a method in the range where the CV = 100%, a zero or negative value occurs on the average of only once every 6 measurements. Third, most methods are not used where they are so imprecise; at a CV of 50%, the use of a method would be expected to result in only a 2% rate of false negatives. Under such circumstances, a false negative would rarely be seen in a series of duplicates or triplicates. When it appears occasionally, it would ordinarily be discarded and replaced by a replicate before it is reported. False negatives usually appear when the range of biological interest strains the capabilities of the available analytical methods.

Finally there is a very large difference in the comparability of analytical results in the intralaboratory environment compared to the interlaboratory environment. An excellent example is the data reported by Freisen (11) on the international proficiency study of the determination of aflatoxin at fairly low levels when the frequency distributions of the reported values for each of the samples are compared. The appearance of the distributions changes from approximately normal at the highest mean sample value to a bimodal distribution with the intermediate mean sample values where one peak is at a positive value and the other peak is at zero, and finally, with the very low level values, to a single peak at zero falling off sharply to positive values. No negative values were reported because aflatoxin analysis does not require a blank. A portion of the clear thin layer plate is automatically set at zero in the determination. There is no problem in interpreting the results from the sample with the single highest value, about 14 ppb. In the case of the bimodal distribution, a meaningful value can be assigned for aflatoxin only if the zero values are discarded as representing determinability failures. But from the viewpoint of each laboratory, there is no problem; each laboratory has given an unequivocal result. Only from the point of view of the entire study is it seen that the result consists of 2 answers: zero and a positive value. When the percentages of zero values are plotted as a function of the mean concentration, the resulting curve takes on the appearance of the general CV/concentration curve. Obviously when the distribution function is bimodal, we have pushed

the method beyond its capabilities. Unfortunately, a single laboratory cannot judge capabilities without assistance from other laboratories to obtain a consensus decision. Note that the same problem exists when blank samples are analyzed, since positive values are sometimes reported. Here too, the result from an individual laboratory is unequivocal until it is examined as part of the total interlaboratory environment.

Cost Limitations

The third factor in assessing chemical realities is the matter of cost. Lundell was very much aware of the importance of this factor. He pointed out that there is little profit in custom-made analyses and also that the purchaser cannot check the correctness of the analyses except by buying other analyses. This is even more true of trace analyses. The chemist cannot be sure of the correctness of his values except by preparing or purchasing and analyzing certified reference materials or by obtaining additional independent analyses in order to arrive at the consensus value or to adjust his performance to match the certified value. Since there are few certified materials and many materials to be analyzed, the consensus values usually must be taken as the best values available. This is true, as we have seen, because of the large systematic and random errors associated with trace analysis.

As we go lower in the concentration scale, our relative errors become larger. By necessity, the methods become longer and more complex in order to obtain the cleanup required for measurement and confirmation of identity. These methods require a level of skill and experience which now takes months and in some cases years to attain. Since 1974, when prices of solvents doubled and tripled and some even became unavailable, we have become sensitive to the cost and volume of the reagents used in trace analysis. We have always been sensitive to the price of the instruments used.

In boasting of new and improved techniques for determining lower and lower levels of chemicals in our food and environment, the chemist has rarely mentioned the cost for this achievement in terms of reduced reliability and in terms of actual dollars. We have already indicated the extent of reduced reliability; FDA operates a regulatory program to control aflatoxins with methods with an interlaboratory CV of about 30% not because we want to, but because, in view of perceived risk to public health, we must. In terms of cost, our laboratory recently participated in an interlaboratory study of the method for the determination of dioxins in fish at about 50 ng/kg (ppt). We estimate that our direct costs for salaries and supplies to perform these analyses was about $1000 per determination. The final measurement is made by mass spectrometry. The direct costs of this laboratory are included in the $1000 per determination figure, but the capital cost of the $2,000,000 in instruments, not all of which are required in this determination, is not included. In common with most government programs, the necessary overhead and training costs are not known and therefore are not included in this estimate. If a routine program for analysis of dioxins in fish is ever instituted, perhaps there will be some cost reduction through volume operation, but costs will have to be added for quality assurance and for sample collection. Therefore, the $1000 per analysis figure may be a sufficiently reliable estimate of the cost for this program.

Now the question becomes an economic one: Is the value of the information as to the dioxin content of fish which may be contaminated worth the cost of obtaining it? In this connection it is not remiss to point out that toxicologists have been unable to provide the analytical chemist with a figure below which the presence of dioxin is of "no biological significance." In addition, there is the uncertainty of interpreting animal experiments in relation to humans, particularly in the light of epidemiological evidence. The toxicologist has shifted the responsibility to the chemist by telling him that the molecule is so toxic to test animals that he must determine levels as low as possible. In other words, the technology of analysis, not biological significance, becomes the limiting factor. In the final analysis, neither analytical chemistry nor regulatory toxicology will make the decision. It will be pure economics: Can we afford the price that trace analysis is demanding for giving us information as to the contaminant content of our foods and of our environment in the absence of an ability to interpret that information in terms of biological significance to humans? At the present state of the art, all toxicologists can do is assess the possible added health effects due to the various levels of exposure to these highly toxic contaminants.

Conclusion

In the final analysis, we find that our chemical realities are not really limited by scientific considerations at all, as Lundell predicted. We are

able to make measurements at lower and lower levels, but at the expense of greater unreliability. This greater unreliability in turn is accompanied by increased costs, costs which a cost-conscious program is unlikely to justify in the absence of a demonstrated or even implied biological significance to humans.

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TECHNICAL COMMUNICATIONS

Survey of Infant Foods for *Clostridium botulinum* Spores

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A total of 236 samples of infant foods, including honey, dry cereal, nonfat dry milk, evaporated milk, canned formula, and canned baby food, were collected in the New York City area and tested for the presence of *Clostridium botulinum* spores. Methods for recovery of spores were validated using foods spiked with 4 spores/mL or g. None of the products contained *C. botulinum* spores, indicating that their incidence in these commercial foods is not widespread. This limited study did not identify any food types that could be suspected of being involved in the transmission of infant botulism.

Infant botulism has been actively investigated since 1976 when Pickett et al. (1) reported that the disease had occurred in 2 infants. In both patients, the course and symptoms were consistent with adult botulism, and toxin was identified in stool specimens. As a result of the intensive interest since 1976, over 180 cases have been diagnosed (2). The disease is found in children 3–26 weeks old (3), and unlike botulism in adults, there is no dependence on consumption of foods containing preformed toxin. Infant botulism results from the ingestion of *Clostridium botulinum* spores which colonize in the gut and subsequently produce botulinal toxin in vivo (1, 4). An early study (5) implicated honey as a source of spores in some cases.

To identify other possible sources of spores responsible for the disease, Chin et al. (6) analyzed 555 food and environmental samples collected during studies of infant botulism in California. Samples included commercial formulas, honey, corn syrup, sugar, cereals, baby foods, canned foods, garden soil, and household dust. Except for honey and, as expected, soil and dust, none of the samples contained viable spores. Nine samples (5 honey and 4 soil or dust) contained *C. botulinum*. The type of toxin (A or B) produced by isolates from the sample matched the type produced by isolates from the patient. Although the microbiological methods used for examination of honey were clearly stated, these authors did not specify sample size, sample source, or methods used to test the other food products.

In the present work a number of foods that could be fed to infants less than 1 year old were surveyed for viable *C. botulinum* spores. The foods were purchased from local retail sources. The purpose of the survey was to determine if infant foods in the New York City market area represented a potential threat to infants because of contamination with *C. botulinum*. The methods used for examination of each product type were validated by analysis of samples spiked with low levels of *C. botulinum* spores.

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METHOD

Spores

The strain of *C. botulinum* used to spike samples was a food isolate shown to produce type A toxin by the mouse bioassay. The culture was maintained in cooked meat broth (Difco Laboratories, Detroit, MI). Spores for inoculating products were produced in tubes containing 100 mL cooked meat broth incubated at 35°C for 9 days. Spores were harvested by centrifugation (10 000 × g for 20 min at 4°C), resuspended in absolute alcohol to kill vegetative cells, washed twice in gel-phosphate buffer, and finally resuspended in 60 mL gel-phosphate buffer. A 1 mL sample of the spore suspension was heat-shocked at 80°C for 10 min, serially diluted in gel-phosphate buffer, and plated on liver-veal egg yolk agar (Difco) to determine the concentration of viable spores. The suspension was stored at −10°C until needed.

Food Products

Food samples were purchased from 8 supermarkets in the New York City area. The investigation was limited to 6 product types: honey, dry cereal, nonfat dry milk, evaporated milk, canned infant formula, and canned (glass jars) baby food. The number of units and type of product tested are listed in Table 1.

Table 1. Product types tested

Product	No. varieties	No. manufacturers	No. units tested
Honey	10	7	48
Dry cereal	12	6	87
Nonfat dry milk	1	5	26
Evaporated milk	1	3	15
Canned formula	5	4	40
Canned (glass jars) baby food	4	1	20
Total	33	26	236

Preparation and Analysis of Samples

Except for honey, products were prepared and analyzed by the methods outlined in the *Bacteriological Analytical Manual* (7). Containers were disinfected with 70% ethanol (membrane filtered), thoroughly shaken, and opened for sampling under an HEPA-filtered laminar flow hood. Sample size for most products was 10 mL (liquid) or 10 g (solid) transferred directly into 100 mL cooked meat medium. Prior to inoculation, tubes of medium were steamed for 30 min in an Arnold steam chamber and cooled until they were still hot to the touch (70–85°C). Ten g samples of nonfat dry milk were diluted with 10 mL sterile saline and then added with a pipet to the bottom of tubes of presteamed cooked meat broth. Ten g dry cereal was suspended in 100 mL sterile saline and then 10 mL of the slurry was added to presteamed cooked meat broth. The tubes were incubated for 7 days at 35°C and examined daily for growth. After incubation, samples of culture broth were mixed with an equal volume of absolute alcohol (membrane filtered) and, after 1 h at room temperature, streaked onto liver-veal egg yolk agar. Duplicate plates were incubated aerobically and anaerobically at 35°C for 48 h and examined for growth. Colonies typical for *C. botulinum* (i.e., pearly layer and yellow precipitate) were picked and inoculated into tubes of cooked meat broth. The tubes were incubated at 35°C for 72 h, after which 0.5 mL culture broth was inoculated into 2 mice as a presumptive test for botulinal toxin.

Twenty-five g honey was analyzed by the dialysis-enrichment culture method of Sugiyama et al. (8). In brief, the honey was warmed, mixed, and diluted with sterile water to permit easier handling. The diluted honey was dialyzed against distilled water and then inoculated into a medium containing cooked meat particles and trypticase-peptone-glucose-yeast extract broth. The inoculated medium was heated at 80°C for 25 min and incubated for 7 days at 35°C.

Results and Discussion

Validation of Methods

One product unit representative of each product type listed in Table 1 was inoculated with spores of *C. botulinum* type A. Except for honey, the spore concentration in each product was approximately 4 spores/mL. Honey was inoculated with about 40 spores/mL. Each inoculated product was examined for the presence of *C. botulinum* by the methods described earlier. In every case, typical *C. botulinum* colonies were detected on liver-veal egg yolk agar, and toxigenicity was confirmed by the mouse bioassay. Cereal products and nonfat dry milk contained large numbers of gram-positive spore-forming rods identified as *Bacillus* sp.; however, the low level *C. botulinum* spike was recovered in each of the product types by this procedure.

Sample Analysis

No *C. botulinum* spores were detected in any of the 236 product units tested. Dry foods and honey products contained gram-positive facultative bacilli but no organisms were detected in any of the canned samples. Since a limited number of sample units were tested in relation to the numbers produced, these results do not prove that *C. botulinum* is absent in the product types examined. However, the results indicate that the incidence is probably low and that a widespread problem does not exist. On the basis of this study, we conclude that infant foods in the New York City area are unlikely to be a major source of spores for infant botulism.

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High Pressure Liquid Chromatographic Determination of Capsaicin in Oleoresin and Personal Protection Aerosols

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A simple and accurate high pressure liquid chromatographic method has been developed for determination of capsaicin in oleoresin capsicum and mineral oil-based aerosol formulations intended for personal protection. Capsaicin, the main ingredient responsible for the spicy pungency of various hot peppers and paprika, is extracted with acetonitrile and aliquots are analyzed on a 10 μm C_{18} column at 280 nm with acetonitrile-water (45 + 55) mobile phase. Dihydrocapsaicin is also extracted and is simultaneously quantitated with propiophenone as an internal standard.

Capsaicin is the main ingredient responsible for the spicy pungency of various hot peppers and paprikas (1). Dihydrocapsaicin is present with all capsaicin obtained from natural sources, at approximately 20–25% of the capsaicin concentration (S. Browne, E. M. Chemicals (1981), private communication). A variety of methods for determination of capsaicin in food products include an organoleptic test (1), colorimetry (2–6), UV spectrophotometry (7, 8), thin layer chromatography (9), gas chromatography (10–12), and high pressure liquid chromatography (HPLC) (13). However, no method exists for the determination of capsaicin in materials intended for personal protection.

The HPLC method (13) specifies a mobile phase gradient and fluorescent detection to determine the capsaicinoids. We obtained a sample of standard grade capsaicin and the HPLC method for assay of concentrated standard material. The method provided is similar to one which we developed, except for a different absorbance maximum. Using the absorbance at 229 nm as suggested in the private communication enhances sensitivity, but the analyst must be cautious when working with materials capable of absorbing in that region. We chose a 280 nm detection wavelength because it provides ample sensitivity with no detectable interferences.

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METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Spectra-Physics 8000-A, equipped with Spectra-Physics 8400 variable wavelength detector, recorder or integrator, and 10 μL injection loop, or equivalent. Integrations and calculations were handled by the data system but manual peak height measurements gave identical results.

(b) *Chromatographic column.*—μBondapak C_{18}, 3.9 mm id × 30 cm (Waters Associates, Inc.). Method performs satisfactorily with similar reverse phase columns (Whatman ODS-1, Dupont 5 μm ODS, and Spectra-Physics Lichrosorb 8RPA).

Operating conditions:—Elution solvent, acetonitrile–water (45 + 55) (Fisher Scientific HPLC grade); flow rate, 2 mL/min; temperature, 40°C; injection volume, 10 μL. Elution order is propiophenone (4 min), capsaicin (6 min), and dihydrocapsaicin (8 min) with baseline separation for each component (Figure 1).

(c) *Internal standard solution.*—Weigh propiophenone (Eastman Kodak) into volumetric flask and dilute with acetonitrile to give final concentration of 0.3 mg/mL.

(d) *Standard solution.*—Weigh 100 mg capsaicin into 200 mL volumetric flask and dilute to vol-

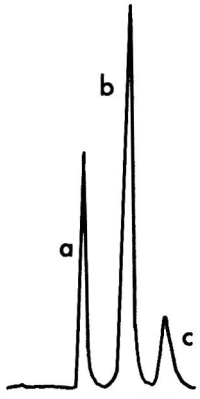

Figure 1. Elution order of a, propiophenone; b, capsaicin; and c, dihydrocapsaicin at 0.26, 0.517, and 0.147 mg/mL, respectively.

ume with the internal standard solution to give final concentration of 0.5 mg/mL.

Preparation of Samples

(a) *Oleoresins.*—Weigh sample which contains ca 25 mg capsaicin into 100 mL screw-cap or glass-stopper Erlenmeyer flask. Add 50 mL internal standard solution and agitate on a mechanical shaker 1 h.

(b) *Aerosol formulations.*—Shake aerosol well, and weigh. Dispense into 100 mL screw-cap or glass-stopper Erlenmeyer flask to obtain sample equivalent to ca 25 mg capsaicin. Add 50 mL internal standard solution and shake 1 h on mechanical shaker. Let sample settle ≥30 min for complete separation of acetonitrile solution from oil layer.

Determination

Inject 2 or more 10 μL aliquots of standard solution to obtain integration parameters and stabilize instrument. Monitor response factor until results agree within 2%. Inject 4 aliquots of standard solution and 2 aliquots of sample solution in succession. Calculate response factor, R, for each:

R = area capsaicin peak/area internal standard peak

$$\% \text{ Capsaicin} = (R/R') \times (W'/W) \times P,$$

where R and R' = average response factors for sample and standard solutions, respectively; W and W' = mg sample and standard, respectively; P = purity (%) of standard.

Results and Discussion

Preliminary investigations established the HPLC conditions, and propiophenone was selected as the internal standard.

For control and recovery studies, a sample of oleoresin capsicum was analyzed, and then used to make a series of spiked solutions containing 0.1–10.0 g oleoresin in a total of 10 g mineral oil (i.e., 1 to 100% oleoresin capsicum in mineral oil). These samples were extracted with the appropriate volume of internal standard solution to yield 0.5 mg capsaicin/mL. Samples were assayed in triplicate and all recoveries were 96–100%, provided at least 3 mL solvent was used per gram of sample. Extraction efficiency was less than 50% when 10 g sample was extracted with 20 mL extractant.

In addition, aerosol products which contained oleoresin capsicum in a mineral oil base and which claimed capsaicin content of about 0.3% were tested. Thus, for the standard solution, a sample of oleoresin capsicum was diluted in mineral oil to yield a calculated content of 0.23%. The prepared dilutions were extracted in duplicate for 1 h in decreasing volumes of acetonitrile (propiophenone added as an internal standard) to test the extraction efficiency in the range used for aerosol sample analysis. The duplicate values were averaged and the results are shown in Table 1.

An 8 g sample in 50 mL extractant, as specified in the method, is within the range of maximum extraction efficiency. Minimum shaking time has not been determined, but recovery studies confirm complete extraction in 1 h. Detection response at 280 nm was linear for 10 μL injections of capsaicin solutions ranging from 0.05 to 1.0 mg/mL. When the internal standard was present at approximately one-half the concen-

Table 1. Efficiency of extraction of capsaicin from mineral oil

Sample, g	Extractant, mL	Capsaicin added, mg	Capsaicin found, mg[a]	Recovery, %
8.66	80	19.92	19.66	98
8.05	60	18.52	18.43	99
7.41	40	17.04	16.89	99
7.35	20	16.90	16.32	96

[a] Average of duplicate analyses.

tration of the standard, peak area ratios were constant over the 0.05–1.0 mg/mL range.

All standards of capsaicin derived from natural sources appear to contain dihydrocapsaicin at 15–20%. Similar proportions of dihydrocapsaicin are found in our samples. The synthetic capsaicin (99% purity) contained none of the dihydro material, and the dihydro material was not available in pure form; therefore, only approximate percentages of dihydrocapsaicin in the samples were obtained. However, if the dihydrocapsaicin content of natural samples is approximated by assuming an ultraviolet absorbancy equal to capsaicin, then the natural samples may be used as the dihydrocapsaicin standard.

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Effects of Grinding and Storage for One Month on Retention of Vitamin A in Premixes and Mineral Supplements

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Tested standardized procedures for handling premixes and mineral supplements from time of sampling to time of analysis for vitamin A have not been developed, which could account for some unexplained inconsistent and low analytical results. Grinding premix samples and storing them in a freezer for one month had little effect on amount of vitamin A found, but there was a significant loss (about 10%) after storage for one month at room temperature. Results on replicated determinations of vitamin A in unground and ground mineral supplements and on effect of storage were somewhat more variable than for premixes, but only the loss (about 12%) during storage for one month at room temperature was significant.

In recent years, many premixes and mineral supplements used to prepare mixed feeds or for free-choice feeding contain added stabilized vitamin A. Those products also commonly contain 15 or more other ingredients, including vitamins and nonmineral components. Regulatory laboratories have found that those premixes and supplements often contain less vitamin A than claimed, with inconsistent differences among laboratories or among analyses performed at different times. When those problems became even more frequent during the summer a few years ago in the midwest United States, procedures for handling samples before the analyses were suspect.

Field-collected samples might have been left in a vehicle exposed to high temperatures for several days before being brought to the laboratory. Samples might have been placed on a shelf at the receiving area of the laboratory, ground when convenient, and again set on a shelf until analyzed some time later. Such practices might have been acceptable in the days when only proximate principles were determined, but are perhaps inappropriate for monitoring vitamin A contents of registered feed products.

Present AOAC procedures for pre-assay handling of samples containing vitamin A

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Table 1. Percentage of sample passing screen

Product	No. of samples	Condition	Screen size 35	60	80
Premixes	14	unground	57–76[a]	16–36	10–23
		ground[b]	84–97	40–74	22–54
Mineral supplements	9	unground	15–63	3–35	1–22
		ground[c]	63–97	28–93	15–52

[a] Range of percentages in 14 and 9 samples, respectively.
[b] Ground in SHG–75g coffee grinder. A supplier is Markson Scientific, Box 767, Del Mar, CA 92014.
[c] Ground in Wiley mill, intermediate model, coarse screen.

(43.008–43.017) are based on tests in mixed feeds, information in trade publications, and on our experience. Few data have been reported on pre-assay handling of premixes or mineral supplements containing vitamin A. The mineral content in those products is higher than in mixed feeds and foods and might be more destructive of vitamin A.

To gain further information on causes and solutions of the vitamin A analytical problems, we studied effects of grinding the premixes and supplements and storing them for one month.

Procedures

Mineral–vitamin premixes were unpelleted commercial formulations designed to supply the micronutrient requirements when added to mixed feeds at 5 or 10 lb/ton. Mineral supplements were formulated for free-choice feeding or for adding to mixed feeds at 20 to 50 lb/ton. Stabilized vitamin A preparations used in the premixes were commercial products from 3 manufacturers, but sources of those used in mineral supplements could not be determined, nor was information available on age and prior handling of supplements. Premixes were either fresh or not more than 2 months old, except one premix was 6 months old. Two of the premixes were listed as containing antioxidant. Contents of vitamin A in IU/g ranged from 1200 to 2000 in 8 samples, 2000 to 3000 in 4 samples, about 5000 in one, and 15 000 in another.

Approximately 40 g portions of the premixes were ground 30 s in a coffee grinder or pulverizer, and portions were combined and mixed. All mixing was done by rolling on paper. Mineral supplement samples (approx. 250 g) were ground in a Wiley mill, an intermediate model containing a coarse screen. The coffee grinder had several advantages over other grinders: fast reduction of particle size, practically no dusting, little heating of sample, ease of cleaning, and relatively inexpensive. However, blades break easily on attempts to grind pellets or large particles. The AOAC method **43.008–43.017** was used to determine vitamin A in 10 or 15 g samples. Chromatography was not required because samples contained little or no carotenoid pigments. Normal laboratory practices were followed for premixes, but work was divided so that each of 2 analysts always did certain parts of the procedure. One analyst did practically all of the work on the mineral supplements.

Results and Discussion

Table 1 shows the particle size distributions in samples. Particle sizes of mineral supplements varied more than those of premixes; some were much coarser. Generally, even after grinding, coarser samples still contained higher percentages of larger particles.

Results on vitamin A contents are shown in Table 2. Overall agreement on triplicate determinations on premixes was entirely satisfactory. Grinding of premixes had little effect on vitamin A contents at 0 time or after freezer storage for 1 month, but storage of either unground or ground samples at room temperature for 1 month resulted in a significant loss of vitamin A. However, grinding of samples did increase (nonsignificantly) uniformity of results on replicated determinations. Satisfactory uniformity in vitamin A determinations was obtained on these samples in which approximately 50% passed a No. 35 screen and 15% or more passed a No. 60 screen.

Storage of mineral supplements at room temperature for 1 month also resulted in a significant loss of vitamin A (Table 2). The larger overall difference (3.6%) and less uniformity in ground and unground mineral supplements than with premixes could have been caused by one or more of the following factors: less uniform mixing by supplier, lower concentrations of vitamin A,

Table 2. Vitamin A content, IU/g

Product	No. of samples	Storage time	Temp.	Unground	Loss, %	Ground	Loss, %
Premixes	14	0		3044 (2.8)[a]		3052 (2.1)	
	8	0		3969 (3.3)		3979 (1.7)	
	8	1 mo.	freezer	3886 (3.4)	2.1	4000 (2.9)	(0.5)[b]
	8	1 mo.	room[c]	3648 (3.3)	8.1*	3567 (1.8)	10.4*
Mineral supplements	12	0	room[d]	223 (4.2)		211 (3.9)	3.6
	13	0	room[d]			316 (5.4)	
	13	1 mo.				279 (3.2)	11.7*

[a] Mean of all samples and % maximum deviation from the mean of triplicate determinations on individual samples (premixes) and of duplicates (mineral supplements). No outlier data discarded.
[b] Increase.
[c] 29–30°C.
[d] Summer uncontrolled laboratory temperature.
* Effect of storage is significant ($P = 0.05$).

dusting during grinding, greater difficulty in cleaning the mill after grinding certain samples. However, some mineral supplements were so coarse that grinding was necessary for satisfactory sampling. These supplements were not studied under low temperature conditions, but, similar to results on premixes, little loss of vitamin A resulted in other mineral supplements stored in a freezer.

The effect of humidity on loss of vitamin A is another factor that is now under study and probably will need to be taken into account along with temperature and time of storage.

These studies indicate that, if the analysis is to reflect the vitamin A content at the time the samples are taken, premixes and mineral supplements should be kept in a freezer until analyzed for vitamin A.

FOR YOUR INFORMATION

AOAC to Hold 97th Annual International Meeting October 2-6

The Association of Official Analytical Chemists (AOAC) will hold its 97th Annual International Meeting October 2-6, 1983 at the Shoreham Hotel, Washington, DC. Current developments in analytical methodology pertaining to agricultural, environmental, and public health areas will be presented and discussed.

Seven symposia planned for this meeting are: Instrumental Methods for the Analysis of Vitamins (Monday, October 3rd); Hospital Disinfectants Testing (Tuesday, October 4th); Food Microbiology: Update on Foodborne Pathogens of Recent Significance (Tuesday, October 4th); Detection/Quantitation of Protein Adulteration in Food (Tuesday, October 4th); Mutagenicity Screening—Microbiological Systems (Wednesday, October 5th); Instrumental Methods and Data Handling (Thursday, October 6th); and Sulfite Analysis Methods for Food Products (Thursday, October 6th).

Approximately 1,200 scientists and researchers from government, industry, and academia are expected to attend. Two hundred papers on new techniques, methods and instrumentation for analysis of foods, drugs, pesticides, cosmetics, feeds, fertilizers, mycotoxins, beverages, colors, forensic science materials, hazardous substances, vitamins, water and air pollutants, and microbiological and extraneous material contamination of foods, and related subjects are to be presented.

Immediately preceding the AOAC meeting on September 30th-October 1st, 1983, the American Chemical Society (ACS) will offer a short course entitled, "Maintaining and Troubleshooting Chromatographic Systems," conducted by M. T. T. Bradley. The fee for the course will be $425.00 for ACS members and $495.00 for non-members. To register for this course, contact the Department of Continuing Education, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036.

AOAC will also sponsor an exhibition of the latest scientific equipment and supplies. Seventy-five firms from all over the United States will be exhibiting their equipment from 10:00 a.m. Monday, October 3rd through 12:00 noon on Wednesday, October 5th.

Registration for the meeting will take place from 1:00 p.m. Sunday, October 2 through 12:00 noon on Thursday, October 6th. The fee for advance registration will be $25.00 (Member)/$30.00 (Nonmember) for one day and $50.00 (Member)/$55.00 (Nonmember) for two or more days. On-site registration will be $60.00 for full meeting and $35.00 for one day.

For hotel reservations, please contact the Shoreham Hotel, Calvert Street and Connecticut Ave. NW, Washington, DC 20008, (202) 234-0700.

For further details, contact Kathleen M. Fominaya, AOAC, 1111 N 19th St., Suite 210, Arlington, VA 22209, (703) 522-3032.

Interim Methods Adopted

The following methods have been adopted interim official first action by approval of the appropriate General Referee, Committee on Official Methods, and Chairman of the Official Methods Board: Mass Spectrometric Determination of Cane Sugar and Corn Syrup in Maple Syrup by Use of $^{13}C/^{12}C$ Ratio, by Mariafranca Morselli and Kelly Baggett, Vermont Agricultural Experiment Station; Determination of Hydrocortisone in Drug Substance and Tablets, by Milda J. Walters, Food and Drug Administration; High Pressure Liquid Chromatographic Determination of Methocarbamol in Pharmaceutical Dosage Forms, by Richard L. Everett, Food and Drug Administration; Determination of Water in Oils and Fats by the Karl Fischer Method, by Raffaele Bernetti, Stephanie J. Kochan, and James J. Pienkowski, CPC International; and Multiresidue Method for Determining Organochlorine Pesticides in Poultry Fat, by James A. Ault and Tim E. Spurgeon, Analytical Bio-Chemistry Laboratories, Inc.

Meetings

October 2-6, 1983: 97th AOAC Annual International Meeting, Shoreham Hotel, Washington, DC. For information, see article above.

April 29-May 2, 1984: 9th AOAC Annual Spring Training Workshop, Philadelphia Marriott Hotel, Philadelphia, PA. Contact Aaron E. Wasserman, PO Box 6197, Philadelphia, PA 19115 (215/676-8568); James J. Karr, Penwalt Technological Center, 900

First Ave, Box C, King of Prussia, PA 19406 (215/265-3200); or Harvey Miller, FDA, US Customhouse, 2nd & Chestnut St., Philadelphia, PA 19106 (215/597-4375).

May 15-18, 1984: 5th International Symposium on Mass Spectrometry in Life Sciences, Gent, Belgium. Contact A. De Leenheer, Symposium Chairman, Laboratoria Voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000, Ghent, Belgium.

October 28-November 1, 1984: 98th AOAC Annual International Meeting, Shoreham Hotel, Washington, DC. Contact Kathleen Fominaya, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209 (703/522-3032).

AOAC Gains New Sustaining Members

AOAC welcomes six new additions to the growing list of companies aware of the need to support an independent methods validation association. New Private Sustaining Members are Borden, Inc., Columbus, OH; Campbell Taggart, Dallas, TX; Carnation, Van Nuys, CA; Comibassal International, Alexandria, Arab Republic of Egypt; Philip Morris USA, Richmond, VA; and Union Carbide Agricultural Products Co., Research Triangle Park, NC.

AOAC also welcomes the Association of Public Analysts, Matlock, Derbyshire, England, as a new Sustaining Member.

ISO Standards Published

The following standards have been published by the International Organization for Standardization (ISO), Technical Committee 34—Agricultural Food Products. The standards are available at prices indicated from the Food and Drug Administration, Bureau of Foods, HFF-7, 200 C Street, SW, Washington, DC 20204.

ISO 5223-1983	Test sieves for cereals	$11.00
ISO 5559-1983	Dehydrated onion—Specification	$12.00
ISO 5560-1983	Dehydrated garlic—Specification	$12.00
ISO 5562-1983	Turmeric, whole or ground (powdered)—Specification	$11.00
ISO 6662-1983	Plums—Guide to cold storage	$11.00
ISO 6663-1983	Garlic—Guide to cold storage	$11.00
ISO 6664-1983	Bilberries and blueberries—Guide to cold storage	$11.00
ISO 6665-1983	Strawberries—Guide to cold storage	$11.00
ISO 6799-1983	Animal and vegetable fats and oils—Determination of composition of the sterol fraction—Method by gas-liquid chromatography	$12.00

1983 AOAC Scholarship Awarded to Bradley Brock of Malvern, PA

Bradley M. Brock, an outstanding student at the Delaware County Campus of the Pennsylvania State University, Media, PA, is the winner of a two-year, $1,000 scholarship sponsored by AOAC.

A Dean's List student with a 3.94 grade point average, Mr. Brock has shown unusual ability in laboratory work and has carried out a Chemistry Honors project. In addition to maintaining a superior academic record and working to finance his education, Mr. Brock has assumed a leadership position in a number of campus organizations.

Mr. Brock will continue his studies at the main campus of the Pennsylvania State University as a biochemistry major with emphasis on human and animal health. He plans to attend graduate school and then teach or carry out research in this field.

Each year AOAC awards a two-year scholarship to a college sophomore who is studying a subject important to public health and agriculture. To qualify, the student must be in need of financial aid, maintain at least a B average during the first two years of undergraduate study, and plan to do research, regulatory work, quality control, or teach in an area of interest to the AOAC.

Nominations for the 1984 award must be received before 1 May 1984. Send six copies of a nomination letter and two supporting reference letters to AOAC, 1111 N 19th St, Arlington, VA 22209.

1983 Fellows of the AOAC

Eight scientists have been chosen to receive the 1983 Fellow of the AOAC awards. Warren Bontoyan, AOAC President, will make the presentations at the opening session of the 97th Annual International Meeting of the AOAC, on Monday, October 3, 1983.

The award recipients are Ted M. Hopes,

Food and Drug Administration, Brooklyn, NY; Edmond J. Baratta, Food and Drug Administration, Winchester, MA; Randolph H. Dyer, Bureau of Alcohol, Tobacco and Firearms, Rockville, MD; Peter M. Scott, Health and Welfare Canada, Ottawa, Ontario, Canada; Alan R. Hanks, Purdue University, West Lafayette, IN; Edwin R. Jackson, State Chemical Laboratory, Mississippi State, MS; Frank J. Johnson, Tennessee Valley Authority, Muscle Shoals, AL; and Stanley M. Cichowicz, Food and Drug Administration, Washington, DC.

The Fellow of the AOAC award was established in 1961 to recognize those persons giving meritorious service to the Association. Winners of the award have performed notably for 10 years or more, usually as officers, referees, or committee members. Nominations are made by AOAC members, reviewed by the Committee on the Fellows, and finally approved by the Board of Directors.

Ted M. Hopes served as Associate Referee on Polarographic Methods for Organomercurial Drugs in the past, member of the Committee on Classification of Methods, and has been the General Referee on Miscellaneous Drugs since 1967.

Edmond J. Baratta previously served as Associate Referee on 3 topics and is presently the Associate Referee on Cesium and the General Referee on Radioactivity.

Randolph Dyer served as Associate Referee on Methyl Alcohol, and is presently the Associate Referee on β-Asarone, Coumarin in Wine, and the General Referee on Alcoholic Beverages.

Peter M. Scott served as AOAC Representative on the Joint Mycotoxin Committee, as Associate Referee on 2 topics, and is presently the General Referee on Mycotoxins.

Alan R. Hanks held 2 Associate Refereeships, 2 General Refereeships, and served on 4 committees. He presently holds an Associate Refereeship on Pentachloronitrobenzene (Pesticide Formulations: Fungicides and Disinfectants) and continues to serve as a member of Committee A and the Editorial Board.

Edwin R. Jackson served as Associate Referee on 5 topics, a General Referee on one topic, and a member on one committee. He currently serves as the Associate Referee on Parathion and Methyl Parathion and is the General Referee on Pesticides Formulations: Organophosphorus Pesticides.

Frank J. Johnson served as the Associate Referee on Mechanical Analysis for Agricultural Liming Materials, a member of Committee A, and the AOAC Representative to ISO/TC 134. He is presently serving on the Board of Directors and is the Associate Referee on Phosphorus in Fertilizers.

Stanley Cichowicz served as a member of the Committee on Meeting Arrangements, was Associate Referee on 4 topics, of which he is presently the Associate Referee on 2, and is the General Referee on Analytical Mycology of Food and Drugs.

CORRECTION

J. Assoc. Off. Anal. Chem. (1983) **66,** 606–609, "High Performance Liquid Chromatographic Determination of Caffeine in Decaffeinated Coffee, Tea, and Beverage Products," by Samy H. Ashoor, George J. Seperich, Woodrow C. Monte, and Jim Welty. In Table 1, the caffeine content of beverages is reported as mg/100 mL, rather than mg/g as in coffee and tea.

Corrected
A-1
26128

NEW PUBLICATIONS

Focusing on Chemistry. Edited by Sybil P. Parker. Published by McGraw-Hill Book Company, 1221 Avenue of the Americas, New York, NY 10020, 1982. 1,195 pp. Price: $49.50. ISBN 0-07-045484-1.

Presenting a technical overview of the main branches of modern theoretical chemistry, the encyclopedia covers all aspects of analytical, inorganic, organic, and physical chemistry. The 790 individual articles explore the nature of matter from the perspective of atomic physics, quantum theory, statistical mechanics, and thermodynamics. The subject matter of the encyclopedia's entries ranges from nomenclature and the elements to sophisticated theoretical concepts such as molecular orbital theory, with detailed discussions on flame photometry, electron spectroscopy, aromatic hydrocarbon, homogeneous catalysis, high pressure chemistry, coordination chemistry, optical activity, halogenation, solid state chemistry, and chemical bond theory. The encyclopedia is illustrated with 800 photographs and line drawings. It also provides cross-references, bibliographies, and a detailed index.

Chromatography in Biochemistry, Medicine and Environmental Research. Proceedings of the 1st International Symposium on Chromatography in Biochemistry, Medicine and Environmental Research, Venice, June 16–17, 1981. Edited by A. Frigerio. Published by Elsevier Scientific Publishing Co., PO Box 211, 1000 AE Amsterdam, The Netherlands, 1983. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017. 278 pp. Price: U.S. $72.25/Dfl. $170.00. ISBN 0-444-42016-9.

The symposium on which this book is based was devoted to the use of chromatography in the biochemical, medical, and environmental sciences. The papers give a variety of examples to illustrate the potential applications of chromatography. Included are studies concerning the identification of drugs, drug metabolism, and pollutants. Research workers in clinical chemistry, biochemistry, medicine, toxicology, and the forensic sciences should find this book of interest.

Clinical Research Practices and Drug Regulatory Affairs. Volume 1. Editor-in-Chief, Gary M. Matoren. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016. Published quarterly beginning 1983. Subscription price per volume (4 issues): $75, institutional rate; $37.50, individual rate.

The clinical research phase of a new drug is vital to that drug's marketing success. This new journal covers the latest advances, findings, and innovations of clinical research practices in the pharmaceutical industry. Topics include experimental design, monitoring practices, computer applications, administration of clinical research programs, quality assurance of clinical research, and the economics of drug development. The journal is aimed toward clinical monitors, clinical information scientists, biometricians, drug regulatory personnel, research administrators, market research staff, and clinical pharmacists, among others.

Comprehensive Analytical Chemistry. Volume XVII: Gas and Liquid Analyzers. By J. Váňa. Published by Elsevier Scientific Publishing Co., PO Box 211, 1000 AE Amsterdam, The Netherlands, 1983. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017. 774 pp. Price: U.S. $170.25/Dfl. $400.00. ISBN 0-444-99691-5.

The book reviews the problems involved in automatic analysis of the chemical composition of substances. Beginning with theoretical background, the author covers the function, technical parameters, and possible application of individual types of analyzer. Examples are given of the use of analyzers in automated production control of the composition of raw materials, in analyses of water and the atmosphere, and as alarms in dangerous environments. The book is a useful aid for workers in measuring and automation departments or control laboratories, as well as for researchers who need information on new branches in analysis.

Methods in Food and Dairy Microbiology. By Leo R. Diliello. Published by AVI Publishing Co., PO Box 831, Westport, CT

06881, 1982. 164 pp. Price: $16.50, outside U.S. $18.00. ISBN 0-87055-411-5.

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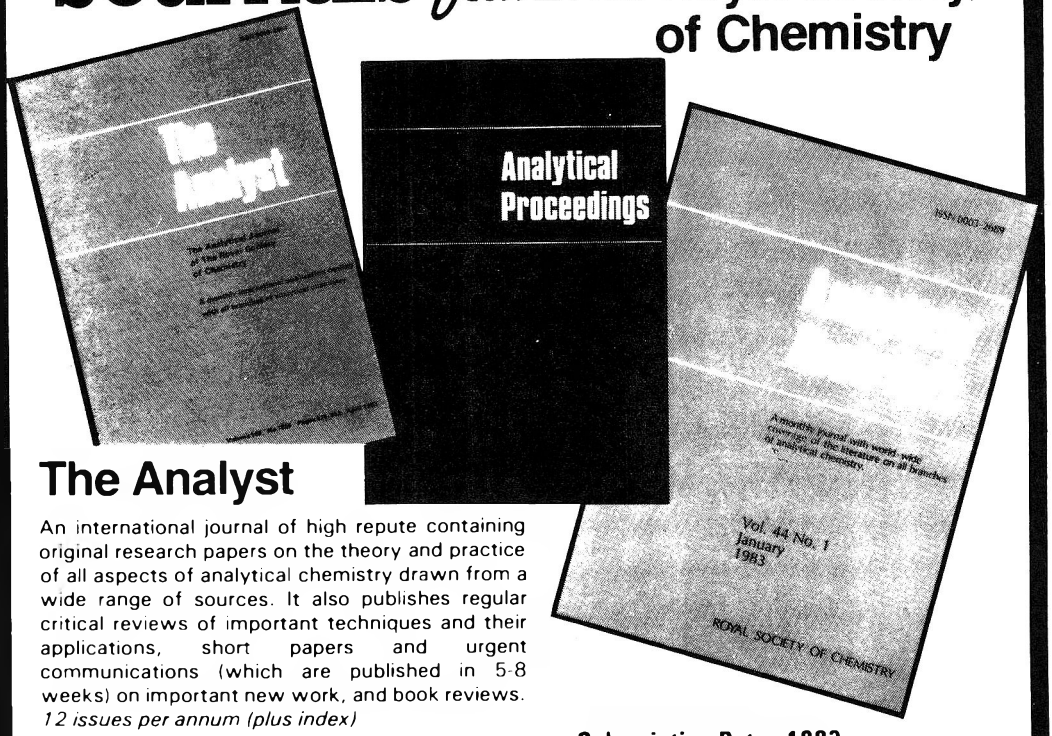

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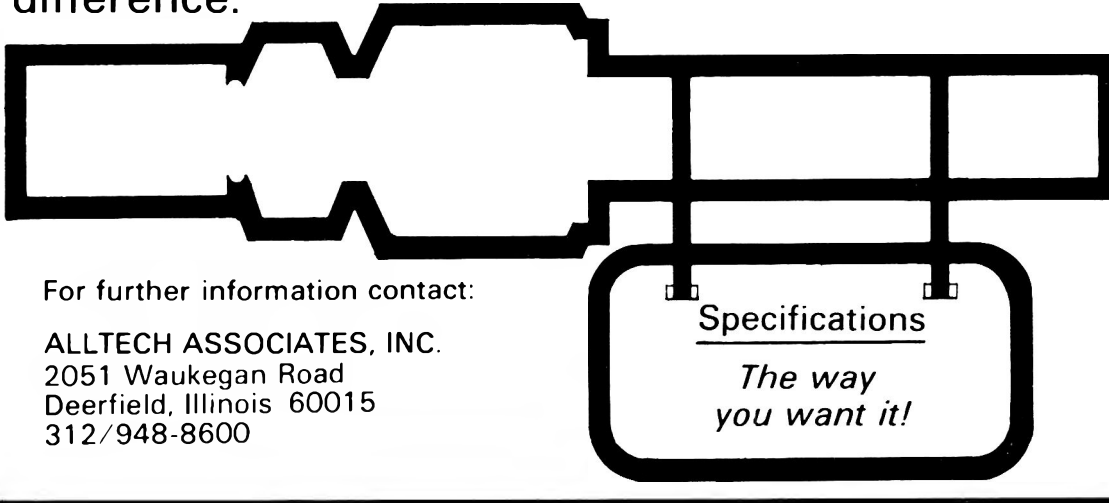

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