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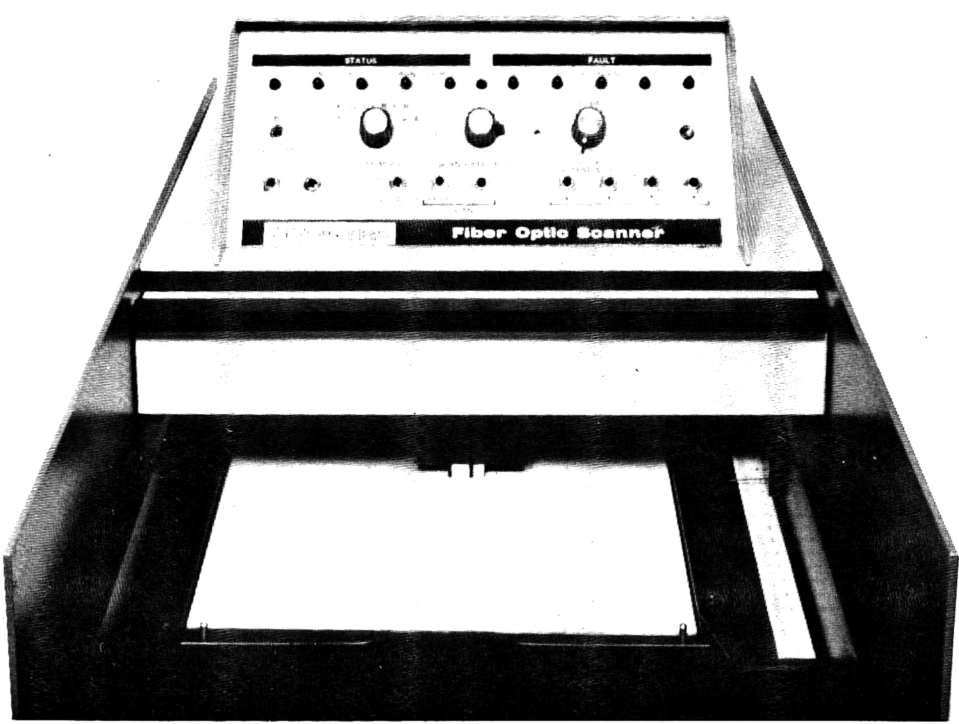

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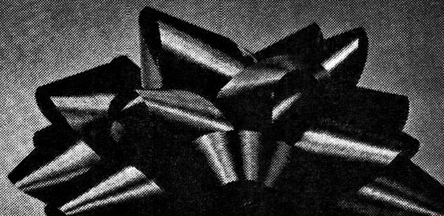

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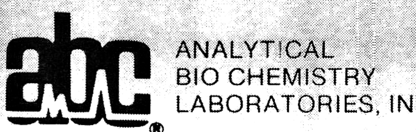

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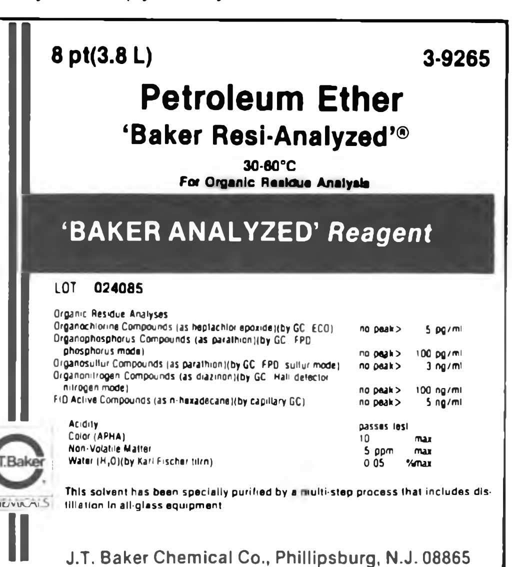
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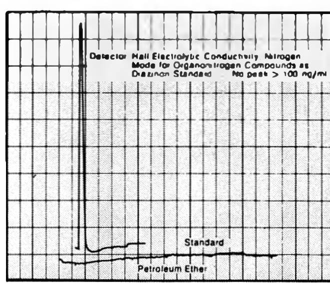

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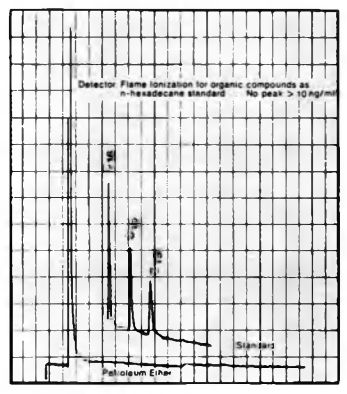

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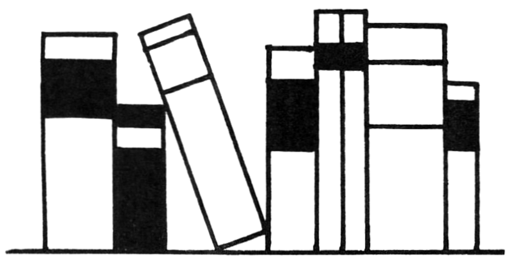

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The abbreviation for the journal title should be repeated for each reference; do not use *ibid*. *This Journal* will be referred to as *J. Assoc. Off. Anal. Chem.*

The compendium of methods of the Association should be listed as follows: *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, with appropriate section numbers; the edition and year are, of course, subject to change.

Symbols and Abbreviations

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

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

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FEEDS

Examination of the Use of Glass Filter Paper in Asbestos-Free Fiber Determination

RICHARD C. CRADDICK and LINDA HATFIELD
Kent Feeds, Inc., Quality Control Laboratory, Muscatine, IA 52761

A modification is presented of asbestos-free method 7.066 for the determination of crude fiber in feeds. In the final filtering step, the fritted glass crucible is replaced with a 7.5 cm disc of glass filter paper in a California Buchner funnel. The data supporting the use of filter paper indicate that good precision and accuracy can be achieved.

With the increasing concern over the carcinogenic nature of asbestos and its use, as well as the inability to purchase the material for crude fiber analysis, we reviewed the asbestos-free method, 7.066, *Offical Methods of Analysis*, 13th edition. Two areas of difficulty in the procedure were recognized, both related to the use of the fritted glass crucible. The crucible is susceptible to stress and breakage when subjected to 600°C temperatures. Slow cooling of the crucible to 250°C before removal from the furnace, or ashing for 3 h at a lower temperature of 500°C, are the suggested alternatives, but both increase analysis time. In addition, the fritted glass filter must be cleaned frequently to avoid slow filtration.

Substituting glass fiber filter paper for the crucible circumvents these difficulties.

METHOD

Principle

See **7.061**.

Apparatus and Reagents

See reagents **7.062**(a, b, d, e, f); apparatus **7.063**(a, b, c, d, e, f) and **14.088**; and in addition:

Glass fiber filter paper.—7.5 cm diameter (Schleicher & Schuell Inc., or equivalent).

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Determination

Extract 2 g ground material with ethyl ether or petroleum ether, **14.088**. If fat is <1%, extraction may be omitted. Transfer to 600 mL reflux beaker, avoiding fiber contamination from paper or brush. Add 0.25–0.5 g bumping granules, followed by 200 mL near-boiling 1.25% H_2SO_4 solution. Place beakers on digestion apparatus at 5 min intervals and boil exactly 30 min, rotating beakers periodically to keep solids from adhering to sides. Near end of refluxing, place California Buchner, **7.063(d)**, previously fitted with No. 9 rubber stopper to provide vacuum seal, into filtration apparatus, and adjust vacuum to about 25 mm Hg (735 mm pressure). At the end of refluxing, decant liquid through funnel, washing solids into funnel with minimum amount of near-boiling water. Filter to dryness, using 25 mm vacuum, and wash residue with four 40–50 mL portions of near-boiling water, filtering after each washing. Do not add wash to funnel under vacuum.

Wash residue from funnel into reflux beaker with near-boiling 1.25% NaOH solution. Add additional hot 1.25% NaOH to provide volume of 200 mL in beaker. Place glass fiber filter paper on the California Buchner funnel screen. At end of refluxing, decant liquid through Buchner funnel and wash solids into funnel with near-boiling water. Increase vacuum as needed to maintain filtration rate. Wash residue once with 25–30 mL near-boiling 1.25% H_2SO_4 solution and then with two 25–30 mL portions of near-boiling water, filtering after each washing. Wash with 25 mL methanol. Break suction, and remove mat by blowing back through filter screen into ashing dish.

Dry crucible with residue 2 h at 130 ± 2°C or overnight, cool in desiccator, and weigh. Ash 30 min at 600°C, cool in desiccator, and weigh.

% Crude fiber = loss in wt on ignition × 100/wt sample

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Table 1. Comparison of crude fiber data (%) of asbestos-free method and collaborative asbestos data on AAFCO check samples

	Asbestos-free		AAFCO-asbestos	
Sample	Mean	SD	Mean	SD
Pig feed medicated	3.02[a]	0.08	3.22	0.33
Broiler starter medicated	1.89[b]	0.03	2.04	0.19
Soybean meal	6.56[a]	0.12	6.54	0.36
Cattle feed	15.62[a]	0.35	15.31	1.60
Dog food expanded	2.17[a]	0.06	2.73	0.37
Beef supplement medicated	3.80[b]	0.08	4.06	0.36
Broiler finisher	1.56[a]	0.02	1.81	0.14

[a] Mean of 6 determinations.
[b] Mean of 5 determinations.

Table 2. Precision of analyses by asbestos-free method on a single sample over consecutive sets of assays[a]

Trial	% Fiber	Average
1 A	2.99	
B	2.98	2.99
2 A	3.04	
B	2.95	3.00
3 A	3.45	
B	3.57	3.51
4 A	2.88	
B	2.83	2.86
5 A	3.06	
B	2.97	3.02
Trials	10	
Average	3.07	
Std dev.	0.24	
CV, %	7.88	
% Range	0.74	

[a] Collaborative AAFCO crude fiber average was 3.22%.

Results and Discussion

To illustrate the accuracy and precision of this modification, we analyzed 7 American Association of Feed Control Officials Check Samples (Table 1). Five duplicate analyses of a single sample were conducted by the asbestos-free method on consecutive sets of assays to observe reproducibility of the method (Table 2). Statistical evaluation of the data indicates good precision and satisfactory comparison with grand average of check samples.

Blank response for the asbestos-free procedure is negligible. We did not observe alteration of glass fiber filter paper due to temperature in the trials undertaken. We have analyzed a variety of feeds and ingredients over a wide range of crude fiber levels and have not encountered difficulties in filtering.

Automatic Karl Fischer Titration of Moisture in Grain

FRANK E. JONES and CARROLL S. BRICKENKAMP[1]
National Bureau of Standards, National Engineering Laboratory, Washington, DC 20234

Automatic Karl Fischer titrators of the motor-driven buret type and the coulometric generation type were applied to the determination of moisture in grain. Techniques were developed to optimize the performance of the Karl Fischer titration method and to overcome disadvantages attributed to it. The imprecision (1 standard deviation) of a determination is typically 0.06% moisture content at a moisture content of 15%; the systematic uncertainty from known sources is estimated to be ±0.05% moisture content at a moisture content level of 15%. It is suggested that the method be designated as the primary reference method for grain in general and for corn and soybeans in particular.

The Karl Fischer titration method (1) for the determination of water was used by Fosnot and Haman (2) in a preliminary investigation of its application to the determination of moisture in cereal grains (wheat and barley) and cereal products. Hart and Neustadt (3) proposed the use of the method for grain to avoid some of the disadvantages of oven methods and adapted it to test the accuracy of official oven methods. U. S. Department of Agriculture (USDA) oven methods have been designed to give results which agree with those obtained by the Karl Fischer (KF) method (4) although a satisfactory oven method has not been developed for soybeans (5). It has been reported (6) that the official USDA oven method for whole corn, 103°C for 72 h, gives values about 1% moisture content too low for the very hard corn grown in France (similar to flint corn grown in the United States).

In spite of the advantages of the KF method, particularly its specificity for water and its demonstrated applicability to the determination of moisture in grain, it has not been designated as the primary reference method. Presumably this is due in part to observations that the method is lengthy and requires considerable technical skill and experience for accurate, reliable results.

However, several significant developments which have overcome disadvantages attributed to the method, particularly with respect to the technical training and requisite skill of operators, are electrometric end-point detection (7) which eliminates the difficult visual detection of the end-point; automatic dispensing of KF reagent from a buret (8); and coulometric generation of iodine (9, 10) which eliminates the buret. As a result of these developments, 2 types of automatic KF titrators are commercially available, both with electrometric end-point detection: titrators with motor-driven burets, and titrators with coulometric generation of iodine.

In the present study, techniques have been developed to apply automatic KF titration to the determination of the moisture content of grain and to overcome disadvantages attributed to the KF method. Also, the estimated uncertainties in the determination have been analyzed, and the results of the analysis have been compared with results for experimental determinations.

METHOD

Apparatus

(**a**) *Automatic Karl Fischer titrator.*—Coulometric Aquatest II (Photovolt Corp., New York, NY 10010), or equivalent; or motorized Beckman Model KF-4B (Beckman Instruments, Inc., Fullerton, CA 92634), or equivalent.

(**b**) *Mill.*—Model 8000 Mixer/Mill with 65 mL capacity hardened steel vessel and a supply of 0.5 in. diameter steel balls (Spex Industries, Metuchen, NJ 08840), or equivalent; and, if desired, mill with a larger capacity vessel to accommodate larger samples.

(**c**) *Analytical balances.*—Model B6 semimicrobalance (Mettler Instrument Corp., Hightstown, NJ 08520), or equivalent; and Model M5 microbalance (Mettler Instrument Corp.).

(**d**) *Syringes.*—1 mL, 2.5 mL, and 10 mL gastight syringes, and 10 μL syringe with 12.7 cm needle (Hamilton Co., Reno, NV 89510), or equivalent.

(**e**) *Test tubes.*—Vacutainer BD-6440, partially evacuated, no additive, no interior coating, pink stopper (Becton, Dickinson and Co., Rutherford, NJ 07070).

(**f**) *Telescopic viewer.*—Made for reading ther-

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[1] National Measurement Laboratory.

Certain commercial equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

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mometers (Parr Instrument Co., Mcline, IL 61265).

(g) *Pipet.*—50 mL Class A.

Reagents

(a) *Karl Fischer reagent.*—Stabilized (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(b) *Karl Fischer reagent diluent.*—Stabilized (J. T. Baker Chemical Co.).

(c) *Vessel solution for Aquatest II.*—Ir. 2 parts (Photovolt Corp.); see revision of ASTM D1533 for preparation.

(d) *Generator solution for Aquatest II.*—Karl Fischer reagent in methanol (Photovolt Corp.).

(e) *Methanol.*—Distilled in glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442).

Sample Preparation and Extraction of Water

Accurately weigh ca 5 g whole grain in capped weighing bottle on analytical balance. Apply air buoyancy correction (11). Put grain and milling balls into milling vessel and pipet 50 mL methanol into vessel. Seal lid to vessel, and mill for predetermined time. Milling time for Spex Mixer/Mill is 15 min, during which time methanol-extracted water mixture is heated by milling to just below boiling point of methanol. Set milling vessel aside to allow solid residue from grain to settle. Allow 1½–2 h if vessel is not centrifuged; centrifuging will greatly reduce settling time. After settling or centrifuging, use 10 mL syringe to remove 10 mL portions of methanol-extracted water mixture from vessel and immediately inject through rubber stoppers of Vacutainers. Dip stoppered end of Vacutainer into melted paraffin to immobilize the stopper and, as a precautionary measure, to further seal hole made in stopper by syringe needle. Repeat above procedure for 50 mL methanol without grain to prepare methanol blank. Minimize exposure of methanol and mixture to air to prevent contamination by moisture in the air.

Before use, rinse milling vessel, lid, and balls with methanol, heat in 80°C air oven for 1 h, and let cool.

Titration

Dismantle calibrated syringe (see section on calibration of syringes) and 10 μL syringe, heat in 80°C air oven for 1 h, let cool. Prepare titrator for titration according to manufacturer's instructions. Withdraw specimen of desired quantity of methanol-extracted water mixture (that which contains about 3 mg water) from Vacutainer into calibrated syringe. Expel this specimen as waste and then withdraw another specimen from Vacutainer, using telescopic viewer to set plunger on desired line on barrel (12). Just before expelling second and succeeding specimens into titration vessel, slosh vessel solution (pretitrated methanol in the case of the motorized-buret type titrator) to flush vessel walls and then let titrator titrate any water picked up from vessel.

Expel specimen into titration vessel and start titration. After end-point has been reached, record titration value (mL of KF reagent used in the case of the motorized-buret type titrator, or indicated mass of water in μg titrated in the case of the coulometric type titrator). For best results, space above vessel solution should be flushed with dry gas. Beckman KF-4B titrator has provision for passing dried air through space continuously. Aquatest II coulometric titrator has no such provision; however, introducing flow of dry nitrogen or dry air through sample port 3–4 s just after sloshing solution is effective.

Repeat titration procedure (except for initial expulsion of specimen as waste) for 2 more specimens. Compute mean of 3 titration values. Then make 3 standardizations as specified in the section on standardization of titrators and compute mean. Then make 3 titrations of methanol blank, and compute mean.

In making a series of titrations in a coulometric titrator, it is necessary to consider the limits on the quantity of water that can be titrated. The manufacturer of the Aquatest II titrator recommends that titration be limited to 1 g in a 2-week period. We found a practical limit of a maximum of about 0.07 g per day. The following is a suggested sequence of titrations for 3 sets of specimens each containing about 3200 μg water, 3 sets of standardizations using about 3200 μg water, and 3 sets of blank determinations: 3 specimen titrations (3S), 3 standardizations (3T), 3S, 3T, 3S, 3T, 3 sets of blank titrations.

Calculations

Calculate moisture content (m.c.), on wet basis, of grain sample, using the following equations:

$$\text{m.c., \%} = (VC/10^6 m_g) \times \{[Z(A_s/v_s) - (A_b/v_b)]/[1 - (ZC/10^6\rho)(A_s/v_s)]\} \times 100$$

$$Z = 1 - 1.985 \times 10^{-7} \times C \times [(A_s/v_s) - (A_b/v_b)]$$

V = volume of methanol used for extraction (mL)

C = standardization factor for the titrator (μg/μg, or μg/mL), mean of 3 values

m_g = mass of grain sample in g

Z = factor which takes account of the fact that the volume of a methanol–water mixture is less than the sum of the volumes the components would occupy separately (dimensionless)

A_s = titration value (μg water for coulometric titrator, or mL KF reagent for motorized-buret titrator) for specimen of methanol-extracted water mixture, mean of 3 values

v_s = volume of specimen (mL)

A_b = titration value (μg water or mL KF reagent) for methanol blank, mean of 3 values

v_b = volume of methanol blank (mL)

ρ = density of water at the temperature at which the measurements are made (g/cc)

For grains with significant oil content, such as corn and soybeans, multiply m.c. calculated above by:

$$1 + (v_o/V)/[1 - (A_b/v_b)/(A_s/v_s)]$$

where v_o is the volume of oil in the methanol-extracted water mixture, estimated as specified in the section on estimation of volume of oil in methanol-extracted water mixture.

Experimental

Calibration of Syringes

To determine the volume of liquid dispensed by a syringe, calibrate syringe by filling with distilled water to appropriate mark on barrel, weigh syringe on analytical balance, then dispense water, and reweigh. For best results, use telescopic viewer to set plunger on appropriate mark (12). Apply air buoyancy corrections for weighings (11). Convert difference in mass to volume by dividing by density of water (13). Make at least 15 determinations and use mean as calibration value.

Standardization of Titrators

Fill syringe of 10 μL capacity to level corresponding to approximate quantity of water to be titrated in specimen and weigh on microbalance. Insert tip of needle beneath surface of solution in titration vessel, expel water, and start titration. Carefully wipe needle clean of solution and reweigh microsyringe. Apply air buoyancy corrections for weighings (11). Mass difference between weighings is mass of water titrated. Standardization factor, C, is the mass (in μg) of water titrated divided by difference in titrator readings, before and after titration.

Estimation of Volume of Oil in Methanol-Extracted Water Mixture

To estimate volume, v_o, of oil extracted into methanol-extracted water mixture from grain with significant oil content, heat 10 mL mixture in weighing bottle in 60°C air oven until residue is apparently dry. Mass of residue, m_r, is mass difference between weight of weighing bottle with residue and weight of empty bottle. Estimate density of residue, ρ_r, from handbook values of densities of relevant oils for particular grain; close estimate is not necessary. Calculate v_o using equation:

$$v_o = (V/10) \times (m_r/\rho_r)$$

where V is volume of methanol used for extraction of water from grain sample.

Derivation of the Moisture Content Calculation Equation

Moisture content (m.c.) on a wet basis of a grain sample is defined as the ratio of the mass of water in the sample to the mass of the sample, expressed as percentage. Mass of water in a sample of mass m_g is equal to $m_g \times$ (m.c./100). Mass of water in methanol used for extraction is equal to $VC \times (A_b/v_b)$, where V is volume of methanol used for extraction, C is standardization factor for titrator, A_b is blank titration value read from titrator, and v_b is volume of methanol used for blank determination. Volume of water in mixture of methanol and water extracted from the grain sample is equal to

$$(VC/10^6\rho) \times (A_b/v_b) + (m_g/\rho) \times (\text{m.c.}/100),$$

where m_g is in g, ρ is density of water in g/cc at temperature at which measurements are made (13), and CA_b is in μg. Total volume of mixture is equal to

$$Z \times [V + (m_g/\rho) \times (\text{m.c.}/100)]$$

where Z is a factor which takes account of the fact that the volume of a methanol–water mixture is less than the sum of the volumes the components would occupy separately (14); in reference 14, Z is designated Z'. Ratio of volume of water in mixture to total volume of mixture is thus:

$$[(VC/10^6) \times (A_b/v_b) + m_g \times (\text{m.c.}/100)]/\{Z \times [\rho V + m_g \times (\text{m.c.}/100)]\}$$

This ratio is also equal to the ratio of volume of water in specimen of mixture to volume of specimen: $(C/10^6\rho) \times (A_s/v_s)$, where A_s is titration value for specimen of volume v_s.

By combining the last 2 expressions and rearranging, the following equation results:

$$\text{m.c.,\%} = (VC/10^6 m_g) \times \{[Z(A_s/v_s) - (A_b/v_b)]/[1 - (ZC/10^6\rho)(A_s/v_s)]\} \times 100 \quad (1)$$

In the present work, $(CA_s/v_s) \leq 30\,000\ \mu g/mL$, $(CA_b/v_b) < 500\ \mu g/mL$, and $Z \geq 0.9940$. Z/ρ is near unity in the temperature range 20–25°C. Therefore, Z/ρ can be set equal to unity in the denominator of Equation *1* with an error of less than 0.00013 of the moisture content.

Z can be calculated in the temperature range 20–25°C, using the equation:

$$Z = 1 - 1.985 \times 10^{-7} \times C \times [(A_s/v_s) - (A_b/v_b)] \quad (2)$$

For grains with significant oil content, such as soybeans and corn, correction must be made to Equation *1* to account for the effect of the volume of oil, v_o, inadvertently extracted into the methanol–water mixture. The total volume of the mixture then becomes approximately

$$Z \times [V + (m_g/\rho) \times (\mathrm{m.c.}/100)] + v_o$$

resulting in addition of the following term to the right hand side of Equation *1*:

$$100 \times (v_oC/10^6\rho m_g)(A_s/v_s)/ \times [1 - (ZC/10^6\rho)(A_s/v_s)]$$

The ratio of this term to the right hand side of Equation *1* (RHS1) is

$$(v_o/V)/\{\rho[Z - (A_b/v_b)/(A_s/v_s)]\}$$

which can be approximated by

$$(v_o/V)/[1 - (A_b/v_b)/(A_s/v_s)]$$

with an error that is negligible compared with the error in the estimation of v_o. Therefore,

$$\mathrm{m.c.,\ \%} = \mathrm{RHS1} \times \{1 + (v_o/V)/[1 - (A_b/v_b)/(A_s/v_s)]\} \quad (3)$$

Uncertainty in Calculation of Moisture Content

To estimate the uncertainty in the calculation of moisture content, the uncertainty in each of the parameters in Equation *1* was estimated and the uncertainties were combined. The measure of random uncertainty used in this work is the estimate of the relative standard deviation of the mean (i.e., the standard deviation of the mean divided by the mean). The measure of systematic uncertainty is the estimate of relative bias. Each of these measures will be referred to as relative uncertainty.

The uncertainty in V, the volume of methanol dispensed from the pipet, consists of a systematic component and a random component. The estimate of the systematic component of the uncertainty in V is the tolerance assigned to the Class A pipet, ±0.05 mL for a 50 mL pipet, for example. The relative systematic uncertainty is ±0.001. The relative random uncertainty is estimated to be 0.0005 from common experience in the use of pipets.

In this work, the typical value of the relative random uncertainty in A_s, the titration value for the specimen of the mixture of methanol and extracted water, determined from 3 replicate titrations, is 0.0028. The relative random uncertainty in A_b, the titration value for the methanol blank, also determined from 3 replicate titrations, is 0.01. The relative uncertainty in v_s, the volume of the specimen of the mixture, determined from 15 determinations of the volume of the syringe filled to a particular graduation on the barrel, is 0.0001 (12). The relative uncertainty in v_b, the volume of the methanol blank, is also 0.0001. These uncertainties in syringe volume are estimates of the relative standard deviation of the mean and are, therefore, random. In use in the determination of moisture content, however, they act as systematic (this treatment is similar to Youden's (15) treatment of random titration error in determining the titer of a volumetric reagent); they have, consequently, been multiplied by 3 and treated as relative systematic uncertainties.

The relative random uncertainty in C, the standardization factor, determined from 3 replicate standardizations, is 0.0031. The standard deviation for the microbalance used to determine the mass of water used in the standardizations is estimated to be 2 μg (R. M. Schoonover, private communication). The resulting uncertainty in the mass of water, considered to be systematic, is 5 μg. The imprecision of the balance used for determining m_g, the mass of the grain sample, corresponds to an uncertainty in m_g of 0.000006 and can, therefore, be neglected. However, if the density of the grain were uncertain by ±0.1 g/cc, the buoyancy correction (11) would be uncertain by approximately ±0.0001 m_g; therefore a relative systematic uncertainty of ±0.0001 will be attributed to m_g. The relative uncertainty in the calculated value of Z is ±0.0005 (14) and is treated here as systematic.

The uncertainty in ρ is that due to the uncertainty in the measurement of the temperature. Because the change in ρ with temperature is about −0.0002 g/cc per °C, the temperature is easily measured sufficiently well to make the uncertainty in ρ negligible.

It should be possible to easily estimate v_o to ±10% relative, the dominant uncertainty being that in the density of the residue.

Table 1. Relative uncertainties for parameters used in calculation of moisture content at 15% moisture content

	Relative uncertainty	
Parameter[a]	Random	Systematic
V	0.0005 (0.008%)	0.001 (±0.015%)
A_s	0.0028 (0.042%)	
A_b	0.0003 (0.004%)	
v_s		0.0003 (±0.004%)
v_b		negligible
m_g	negligible	0.0001 (±0.002%)
C	0.0031 (0.047%)	0.0016 (±0.024%)
Z		0.0005 (±0.008%)
ρ		negligible

[a] $V = 50$ mL; $A_s = 3063\ \mu g$; $A_b = 100\ \mu g$; $v_s = v_b = 0.2$ mL; $m_g = 5$ g; quantity of water for standardization = 3063 μg.

The contributions of the relative uncertainty in each of the parameters in Equation *1* to the uncertainty in calculated m.c. was determined by differentiating the equation with respect to the parameter and relating the relative uncertainty in m.c. to the uncertainty in the parameter.

A calculation of a 5 g sample of grain of 15.00% m.c. was used to estimate the relative uncertainty in m.c. due to the relative uncertainty in each of the parameters. The results are given in Table 1; the corresponding uncertainties in m.c. are given in parentheses.

The random components of the relative uncertainties are combined by using a propagation of error formula (i.e., by quadrature) to estimate the overall random relative uncertainty in m.c. The result is 0.0042 which corresponds to 0.063% m.c. at an m.c. of 15%. The systematic components are combined by addition; the result is ±0.0035 which corresponds to ±0.052% water at 15.00% water. If the grain sample were corn with an oil content of 5%, a relative uncertainty of ±0.0005 (±0.008% m.c.) would be added to the combined systematic relative uncertainty, if all of the oil were extracted into the methanol.

Results for Three Measurements on a Corn Sample

The agreement between independent determinations of the m.c. of subsamples taken from the same grain sample is illustrated by data taken by C. P. Fulmer of the Consumer Protection Division of the South Carolina Department of Agriculture, using the techniques developed in the present work. The volume of methanol used for extraction of water from each of 3 subsamples was 50 mL, and the masses of the subsamples were 6.56540, 5.63876, and 5.43230 g. The results were 14.306, 14.273, and 14.388% m.c. The relative standard deviation for these 3 determinations is 0.0041, corresponding to 0.059% m.c. For these data the overall random relative uncertainty calculated as in the section on uncertainty in calculation of moisture content is 0.0051, corresponding to 0.073% m.c.

Training of Operators

The ease of operation of automatic KF titrators is illustrated by results obtained by personnel having limited formal technical training. Several state weights and measures agencies have been using automatic KF titrators and the techniques presented in this paper in a cooperative program (16) that provided data which are useful in evaluating the KF method as a reference method. After a few hours of instruction and practice, personnel of these agencies, none of whom was a professional chemist, produced results that were as precise as those reported in this paper. In fact, the excellent data for the 3 independent measurements on corn subsamples are results from the program.

Discussion and Conclusions

In the present work, automatic Karl Fischer titrators have been applied to the determination of the moisture content of corn, soybeans, grain sorghum, hard red winter wheat, hard red spring wheat, oats, and rice.

The results reported here have shown the KF method to be precise (standard deviation is typically 0.07% m.c. at an m.c. of 15.00%), easy to apply even by personnel with little or no formal technical training, applicable to a wide range of moisture content, and relatively rapid.

Although the results of Hart and Neustadt (3) and of Weise et al. (17) indicated that in the grains they investigated there are no interferences, i.e., substances that react with either the KF reagent or methanol, it is not possible to make the definitive general statement that there are no interferences in grain. Rather, it would be necessary for particular unknown cases to compare the results for KF titration with those for gas chromatography (17) to determine whether there were differences attributable to interferences.

In the absence of interferences, the KF method can be said to be specific for water. If specific for water, then the method could be used to *define* moisture content. That is, the moisture content of a sample of grain would be defined as the moisture content as determined by KF titration. Uncertainties in the measurements are essentially those discussed in the section on uncertainties, in addition to a possible uncertainty due to incomplete extraction of the water from the

grain. The results of experiments conducted in the present work indicated that the extraction procedures removed effectively all of the water that could be extracted by methanol. The technique of extraction of water using a sealed vessel on a ball mill is superior to other methods of which the authors are aware.

The time required for the titration of a specimen in the coulometric titrator is approximately 5–10 min depending on the amount of water in the specimen; the time required for the titration in the buret-type titrator can be less than in the coulometric titrator. The time required for the titration of the blank can be significantly less, particularly for the coulometric titrator. The preparation of the sample including milling and settling requires about 2 h, or about 45 min if a centrifuge is used for the settling. With planning, taking advantage of the fact that specimens can be stored in sealed test tubes, the number of specimens titrated per day can be optimized. However, the amount of water that can be titrated in the coulometric titrator used in the present work is limited, as noted above. The use of more than one reaction vessel including generator and potentiometric electrodes would increase the number of titrations that could be made per day. The time required for sample preparation and titration is not considered to be restrictive on the use of the KF method as the primary reference method.

The disadvantages of oven-drying methods as primary reference methods for the determination of the moisture content of grain are well known (1, 3, 18, 19); they include the fact that they are *not specific for water* and that the results are affected by vaporization of substances other than water and by chemical reactions. As noted earlier, a satisfactory oven-drying method has not been developed for the determination of moisture in soybeans (5, 17). Proposed primary reference methods involving vacuum desiccation over phosphorus pentoxide (20) at moderate temperatures are slow and it is uncertain whether equilibrium is attained. Gas chromatography is an excellent method for the determination of moisture; however, at present, this method is at a practical disadvantage compared with automatic KF titration because of the higher cost of the gas chromatographic equipment and the requisite skill of operators.

The application of automatic KF titrators to the determination of the moisture content of grain by using the techniques described in this paper has overcome disadvantages attributed to the KF method and has demonstrated its superiority over other methods used as, or proposed as, primary reference methods. Therefore, it is proposed that the Karl Fischer method as applied in this paper be designated the primary reference method for the determination of moisture in grain.

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The authors are grateful for helpful discussions with J. K. Taylor and R. M. Schoonover; and for the contributions of R. J. Smith, formerly of Corn Products, Inc., who made a major contribution by suggesting the use of the Mixer/Mill, C. P. Fulmer, of the Consumer Protection Division of the South Carolina Department of Agriculture, personnel of state weights and measures agencies who contributed data used in the evaluation of automatic KF titration, and Pauline H. Gurewitz who typed the manuscript. The first author is especially grateful for the inspiration and suggestions of V. B. Jones.

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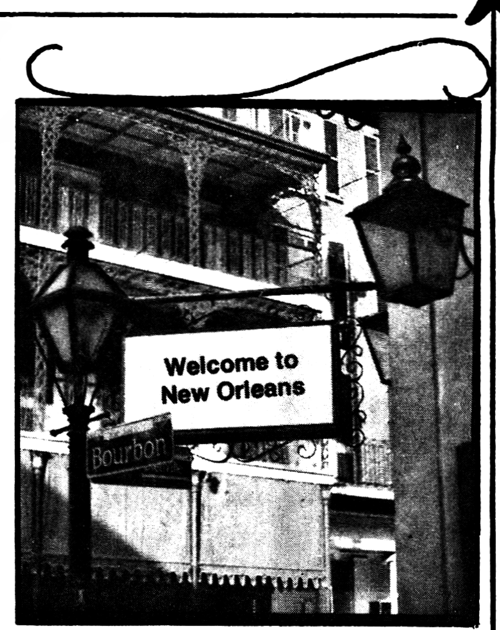

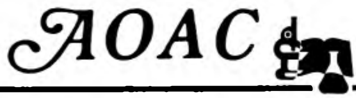

Analysis of Laboratory Animal Feed for Toxic and Essential Elements by Atomic Absorption and Inductively Coupled Argon Plasma Emission Spectrometry

WILLIAM M. BLAKEMORE and STANLEY M. BILLEDEAU
Food and Drug Administration, National Center for Toxicological Research, Jefferson, AR 72079

Analytical procedures are described for the determination of arsenic, cadmium, calcium, copper, lead, mercury, selenium, and zinc in animal feed. Mercury is determined by digesting the feed sample in a mixture of concentrated nitric and sulfuric acids with vanadium pentoxide added as an oxidation catalyst, reducing with stannous chloride, and sweeping the elemental mercury into an absorption tube for measurement by atomic absorption (AA) spectrophotometry. Arsenic and selenium are determined simultaneously by digesting the sample with a mixture of concentrated nitric, sulfuric, and perchloric acids; the hydrides of arsenic and selenium, which are formed with the addition of sodium borohydride, are swept into an argon-hydrogen flame for analysis by AA. A low temperature ash is prepared and dissolved in 1N HNO_3 for the analysis of calcium, copper, and zinc by emission spectroscopy using the inductively coupled argon plasma source; the same solution is used for the determination of cadmium and lead by flameless AA. Animal feed spiked with 3 levels of each of the 8 elements gave recoveries that ranged from 80 to 107%.

Calcium, copper, zinc, arsenic, and selenium are essential nutrients, whereas cadmium, lead, and mercury are considered toxic elements (1). Of all the minerals, calcium appears to be the most intricately involved in the physiological mechanisms responsible for carrying on the life processes; thus calcium needs to be one of the best regulated constituents of extracellular fluid in animals (2). A considerable amount of information about copper is available, pertaining to its absorption, its biochemical role within the cell, and its excretion by various species (3). Zinc deficiencies, which have been observed in a broad spectrum of species, lead to growth inhibition, loss of appetite, and dermatitis (4). Arsenic acts as an essential nutrient by promoting growth and feed efficiency in pigs and poultry but can be very toxic, depending on the amount and chemical form ingested (5); pentavalent arsenic is much less toxic than the trivalent form. Selenium, now considered an essential nutrient that is necessary for growth, fertility, and prevention of disease in animals, was, however, recognized long ago to be toxic (6). The toxicity of mercury in both organic and inorganic forms is well documented (7).

Cold vapor atomic absorption (AA) spectrophotometry is commonly used for determining traces of mercury in a wide variety of samples. Hatch and Ott (8) used stannous chloride to reduce inorganic mercury found in rocks, and Toffaletti and Savory (9) used sodium borohydride to reduce organic and inorganic mercury in urine. A simplified wet digestion method for the determination of mercury in biological samples has been described by Knechtel and Fraser (10).

Methods that use AA (11–13) and inductively coupled argon plasma (ICAP) emission spectrometry (14, 15) have been described for the analysis of aqueous solutions of calcium, copper, and zinc. Procedures have been reported for the determination of cadmium and lead in biological samples by flameless AA (16–18). Bailey et al. (19) used tungsten-coated graphite tubes to reduce matrix interferences in the analysis of blood for lead, and Kitagawa et al. (20) used the Faraday effect to reduce background interferences in the determination of cadmium by atomic fluorescence spectroscopy. A very specific method for the determination of arsenic and selenium in environmental samples involves the formation of the hydrides, which are analyzed by AA in an argon-hydrogen flame. McDaniel et al. (21) used radiotracers to show the efficiency of selenium hydride generation with sodium borohydride; Siemer and Koteel (22) compared various methods of hydride generation and AA detection for arsenic and selenium. However, the procedures described in the literature were not adequate for the determination of these 8 elements in as complex a matrix as that found in animal feed.

All animal feed used in toxicological studies must be analyzed for toxic as well as essential elements, and only those lots that meet the desired specifications can be accepted for use in animal studies. The presence of excessive amounts of toxic elements in the untreated ani-

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mal feed used in bioassays conducted at the National Center for Toxicological Research (NCTR) could bias results obtained with test chemicals, whereas deficiencies of some essential elements might show a biological effect.

Tentative requirements proposed for these 8 elements in animal feed were arsenic, 1.0 ppm max.; cadmium, 0.250 ppm max.; calcium, 0.75% min.; copper, 8.0 ppm min.; lead, 1.5 ppm max.; mercury, 0.200 ppm max.; selenium, 0.05 ppm min. and 0.65 ppm max.; and zinc, 75 ppm min.

Since the initiation of the present study, these maximum allowable concentrations have been revised in "Policies and Procedures for Murine Rodent Diets" (Oct. 31, 1979, amended Jan. 14, 1981). The revised requirements for toxic elements are the same as the tentative requirements for arsenic, cadmium, lead, and selenium; the revised requirement for mercury is 0.1 ppm max.; other elemental concentrations, including those for calcium, copper, and zinc, may be specified by the Principal Investigator for individual experiments.

The methods formerly used at the NCTR required 4 separate dry-ashing procedures plus 2 wet digestions with a mixture of nitric, sulfuric, and perchloric acids. Calcium and zinc were determined by AA in an air-acetylene flame in 1 ashed sample; cadmium and lead were determined by flameless AA in a separate sample; and arsenic and copper were determined in a colorimetric analysis that required 2 additional separate ashed samples. Selenium was determined by spectrophotofluorescence using a wet digestion, and mercury was determined by the cold vapor AA method using a second wet digestion. These methods required approximately 40 man-hours for the analysis of 4 animal feed samples.

This paper describes methodology for proper digestion of animal feed samples to ensure optimum recoveries of these 8 elements in determinations using ICAP and AA. A low temperature ash is prepared for simultaneous ICAP determinations of calcium, copper, and zinc and for simultaneous determinations of cadmium and lead by flameless AA. Arsenic and selenium are determined by generating their respective hydrides from a wet digestion for simultaneous AA detection in an argon-hydrogen flame. An additional wet digestion is prepared for the analysis of mercury by cold vapor AA. The improved methods have decreased analysis time by approximately 30%.

Experimental

Reagents and Apparatus

All reagents were ACS reagent grade.

(a) *Standard stock solutions.*—1 mg analyte/mL. Prepare solutions of the following analytes or their equivalents: arsenic trioxide No. 11100, cadmium No. 20870, copper No. 61143, lead No. 15312, selenium No. 84894, zinc No. 96450 (Tridom Fluka, Mamaronek, NY 11787); anhydrous calcium carbonate (No. C-64, Fisher Scientific Co., Pittsburgh, PA 15219); and mercuric chloride (No. 400216, Ventron Corporation, Danvers, MA 01923). Serially dilute the stock solutions with appropriate aqueous solvents to obtain desired concentrations for standards and spiking solutions.

(b) *Reagent solutions.*—Prepare stannous chloride solution (10 g/100 mL) by diluting solution containing 40 g $SnCl_2$/100 mL concentrated HCl with deionized water. Prepare sodium borohydride solution immediately before use by dissolving 4 g $NaBH_4$ in 100 mL deionized water.

(c) *Animal feed.*—Laboratory chow, type 5010-C (Ralston Purina Co., St. Louis, MO 63188), or equivalent; pH 5.5, 6% fat, and 6.7% volatile after heating at 110°C overnight.

(d) *Apparatus for generation of hydrides and mercury vapor.*—The apparatus shown in Figure 1 was used to generate the hydrides of arsenic and selenium for subsequent AA analysis in an argon-hydrogen flame. The same apparatus was used for the reduction of mercury to its elemental state for AA analysis in an absorption tube. The parts of the apparatus are (*1*) reaction vessel, 125 mL (No. K633030, Kontes Co., Vineland, NJ 08360); (2) Claisen adapter (No. K 161600, Kontes Co.); (3) bushing adapter, size 242 (No. K 150750, Kontes Co.); (4) addition funnel, 25 mL (No. K 299230, Kontes Co.); (5) 3-way stopcock (No. K 822500, Kontes Co.); (6) bubble trap flask, 50 mL (No. 601000, Kontes Co.); (*7*) polyethylene stopper, 24/25 (No. K 853001, Kontes Co.); (*8*) twin Ace thread adapter (No. 5031, Ace Glass, Inc., Vineland, NJ 08360); (9) check valve (No. 120-899, Curtin-Matheson Scientific, Houston, TX 77001); (*10*) drying tube, 100 × 16 mm id (No. 043-869, Curtin-Matheson Scientific). Argon flow was monitored by a rotameter and directed either through the reaction vessel and bubble trap flask or through a bypass directly into the drying tube.

(e) *AA spectrophotometer.*—Instrumentation Laboratory, Inc. (Wilmington, MA 01887) Model

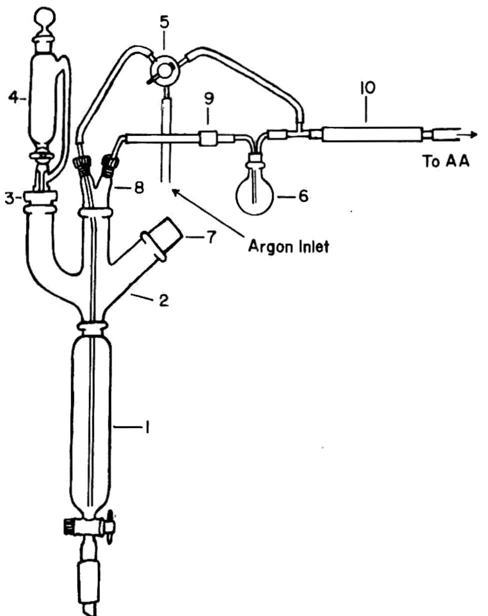

Figure 1. Diagram of apparatus for generation of hydrides and mercury vapor: (1) reaction vessel, (2) Claisen adapter, (3) bushing adapter, (4) addition funnel, (5) 3-way stopcock, (6) bubble trap flask, (7) polyethylene stopper, (8) twin Ace thread adapter, (9) check valve; (10) drying tube 16 mm id × 100 mm long.

751, or equivalent, equipped with 2 double beam channels using two 330 mm Ebert monochromators, deuterium-arc background correction, Model 1L555 controlled-temperature furnace atomizer (Instrumentation Laboratory, Inc.), Model 1L254 automatic sampler (Instrumentation Laboratory, Inc.), and Model 95-8290 dual-pen, flat-bed recorder, or equivalent (Honeywell, Inc., Fort Washington, PA 19034).

(f) *Absorption tube.*—100 × 25 mm id, with quartz windows.

(g) *ICAP emission spectrometer.*—Mark III, or equivalent (Jarrell Ash Div., Fisher Scientific Co., Waltham, MA 02154), equipped with Digital Equipment Corp. (DEC) (Marlboro, MA 01752) central processing unit PDP-11/34, 124K MOS memory, dual RL01 cartridge disks (5.2 megaword), and Jarrell Ash Mark III Sail software.

(h) *Low temperature asher.*—Model PM 248, or equivalent (Dionex Corp., Hayward, CA 94544), equipped with a program controller for automatic operation.

Determination of Calcium, Copper, and Zinc

Accurately weigh 1 g sample into 60 mm diameter petri dish, place dish in reaction chamber of low temperature asher, and set program for 24 h at 50 watts radio frequency power with gas flows of 80 mL/min for oxygen and 20 mL/min for Freon 14 (CF_4). When degradation is complete, dissolve residue in 10 mL 1N HNO_3 with gentle heating, transfer to 25 mL volumetric flask, and dilute to volume with deionized water. Standardize ICAP instrument with aqueous standard containing 200 ppm calcium, 1 ppm copper, and 2 ppm zinc. Adjust acid concentration of standard to that of digested feed sample, and use reagent blank for zero concentrations. Aspirate sample and read concentrations of calcium, copper, and zinc directly, after applying a dilution factor of 25.

Determination of Cadmium and Lead

Use solutions remaining from determination of calcium, copper, and zinc for simultaneous determination of cadmium and lead by flameless AA spectrophotometry with furnace atomizer and autosampler accessories. Aspirate sample solution into graphite tube 20 s at 150°C (aspiration rate 1 μL/s), dry additional 10 s at 150°C, char 20 s at 300°C, and atomize 10 s at 2250°C. Integrate peak heights for 1 s. Determine lead on channel A at λ = 283.3 nm, slit width 320 μm, lamp current 5 mA, with deuterium-arc background correction; determine cadmium on channel B at λ = 228.8 nm, slit width 320 μm, lamp current 7 mA, with deuterium-arc background correction. Determine aqueous standards and reagent blank, and enter values into microcomputer so that concentrations of samples are shown on digital readout for each determination.

Determination of Arsenic and Selenium

Accurately weigh 2 g animal feed into 100 mL micro Kjeldahl flask and add 12 mL concentrated HNO_3. After foaming subsides, add 4 mL concentrated H_2SO_4 and 2 mL $HClO_4$ (70%), and let stand at ambient temperature until reaction ceases. Use micro Kjeldahl digestion unit to heat mixture gently in initial stages to avoid any loss, and slowly increase heat to vigorous boil. Add small amounts of concentrated HNO_3 at first signs of charring. (The solution will turn yellow and then become clear.) Remove flask and swirl contents so they contact bulb area and lower neck of flask. (The yellow color may reappear.) Continue heating until contents are clear, and white fumes appear. (In some instances the solution may remain pale yellow; however, do not continue heating after the white fumes of $HClO_4$ appear.) Remove flask, add 2 mL 30% H_2O_2, and

swirl contents; after all action ceases, continue heating until contents are boiling briskly and white fumes appear. Repeat procedure with 2 additional 2 mL portions of 30% H_2O_2 to ensure that all traces of HNO_3 are removed. Cool flask and carefully transfer contents to hydride-generation apparatus (Figure 1), washing with 45 mL 4N HCl. Turn 3-way valve to let argon at flow rate of 100 mL/min pass through solution, bubble trap flask, and drying tube filled with anhydrous $CaCl_2$ (4-mesh), and into hydrogen-argon flame in AA spectrophotometer. Add 10 mL 4% $NaBH_4$ solution through 25 mL addition funnel to produce the respective hydrides and record absorptions. Determine selenium on channel A at λ = 196.0 nm, slit width 320 μm, lamp current 8 mA, and deuterium-arc background correction. Determine arsenic on channel B at λ = 193.7 nm, slit width 320 μm, lamp current 8 mA, with deuterium-arc background correction. Turn 3-way valve to divert argon flow around reaction flask so the sample solution can be drained and the flask rinsed for the next determination. Determine concentrations by comparing peak heights to those for known amounts of standards, and use reagent blank for zero concentrations. Adjust acid concentrations of standards to make them identical to those of digested samples.

Determination of Mercury

Accurately weigh 2 g animal feed into 100 mL micro Kjeldahl flask, and add 80–100 mg mercury-free V_2O_5, followed by 10 mL concentrated HNO_3 and 15 ml concentrated H_2SO_4. After foaming has subsided, use micro Kjeldahl unit to heat gently in initial stages; slowly increase heat to vigorous boil. When uniform solution is obtained, remove from heat and let cool. Transfer contents to reaction flask of vapor-generation apparatus (Figure 1), using deionized water to wash flask and dilute volume to 100 mL. Turn 3-way valve to let argon at flow rate of 100 mL/min pass through solution, bubble trap flask, and drying tube filled with anhydrous $Mg(ClO_4)_2$, and into absorption tube, which is aligned with the light path in the AA spectrophotometer. Add 10 mL 10% $SnCl_2$ solution from 25 mL addition funnel, and record absorption of mercury vapor at λ = 253.7 nm, slit width 320 μm, lamp current 4 mA, with no background correction. Turn 3-way valve to divert argon flow around reaction flask so the sample solution can be drained and the flask rinsed for the next determination. Determine concentrations by comparing peak heights to those for known amounts of standards, and use reagent blank for zero concentrations. Adjust acid concentrations of standards to make them identical to those of digested samples.

Recovery Studies

(a) *Cadmium, calcium, copper, lead, and zinc.*— Triplicate 1 g samples of animal feed were fortified with 0.1, 0.2, and 0.3 ppm cadmium; 500, 1000, and 1500 ppm calcium; 2.0, 4.0, and 6.0 ppm copper; 0.5, 1.0, and 1.5 ppm lead; and 10, 20, and 30 ppm zinc. A solution was prepared in deionized water with the appropriate amount of each element in admixture so that the addition of 1 mL to the feed sample resulted in the fortification of each element at the lowest level. Solutions for the 2 higher levels were prepared in like manner. The samples were air-dried at ambient conditions before ashing and analyzing as described above.

(b) *Arsenic and selenium.*—Triplicate 2 g samples of animal feed were fortified with 0.1, 0.2, and 0.5 ppm of each element by adding the appropriate amounts of both chemicals for each concentration level in a single 1 mL portion of deionized water. The samples were air-dried at ambient conditions before digestion and analysis as described above.

(c) *Mercury.*—Triplicate 2 g samples of animal feed were fortified with 0.05, 0.1, and 0.2 ppm mercury by adding the appropriate amount of chemical in 1 mL deionized water. The samples were air-dried at ambient conditions before digestion and analysis as described above.

Results and Discussion

Digestion procedures must be carefully chosen to prevent the loss of volatile analytes and the formation of insoluble complexes. In high-temperature ashing methods, calcium may form silicate complexes that are insoluble in acids and require additional digestion for valid analyses. Losses of cadmium and lead may occur in some matrices at temperatures as low as 400°C, especially when chlorides are present. For these reasons, a low-temperature ashing procedure was selected to digest the samples of animal feed for the determination of cadmium, calcium, copper, lead, and zinc.

The ICAP instrument with multiple-element detection and a clean background spectrum is preferred when the concentration of the analyte is within its range of detection. Calcium, copper, and zinc in animal feed were determined simultaneously by ICAP with good precision and specificity. Cadmium and lead were found in

the samples of animal feed at the 0.15–0.25 and 0.5–1.5 ppm levels, respectively. When a feed sample is ashed, dissolved, and diluted (to a concentration of 1 g/25 mL) to reduce matrix interferences, the final solution contains 0.006–0.010 ppm cadmium and 0.020–0.060 ppm lead. Both cadmium and lead can be detected at these levels by ICAP; however, because the precision was poor, regulatory decisions involving samples containing cadmium or lead at levels near the maximum concentration allowed would be questionable. Flameless AA provided good precision and simultaneous detection for both cadmium and lead with minimum detectable levels of 0.001 and 0.010 ppm, respectively, in the final solution.

Precautions must be taken in the preparation of feed samples for arsenic and selenium assays because arsenic trichloride is very volatile (bp 130°C), and selenium distills in various acid vapors. Losses may be minimized by oxidizing to the selenate and to pentavalent arsenic in nitric acid solution. Thus wet digestion with a mixture of concentrated nitric, sulfuric, and perchloric acids was selected for the preparation of animal feed samples used for arsenic and selenium assays. Caution must be exercised in this procedure to ensure that there is no charring of organics during the digestion; otherwise a loss of selenium is likely to occur. Boiling to sulfur trioxide fumes will remove all of the nitric acid present; however, some of the selenium will be lost. Adding hydrogen peroxide and boiling to perchloric acid fumes 3 times ensures complete oxidation of the sample and removes the last traces of nitric acid, which would deter the formation of hydrides if present in the final solution. Preliminary experiments showed that the recovery of arsenic was the same for each of these methods but that considerable selenium was lost when the solution was boiled to sulfur trioxide fumes. Generation of the hydrides, with simultaneous determination of arsenic and selenium in an argon-hydrogen flame by AA, was specific with good precision and minimum detectable levels of 25 ppb for arsenic and 50 ppb for selenium.

The volatility of mercury and its compounds, especially the chlorides, iodides, sulfides, and organics must be considered in sample preparation. This volatilization occurs even in boiling solutions unless provision is made for carrying out the decomposition in flasks with condensers. The long neck of the micro Kjeldahl flask, used in the wet digestion of feed samples for mercury assays, provides an air condenser to minimize these losses. The cold vapor AA technique is based on reducing inorganic mercury salts with stannous chloride and sweeping the free mercury atoms into a quartz absorption tube mounted in the instrument for detection by AA. This method provided good precision, specificity, and a minimum detectable level of 10 ppb for mercury.

Table 1. Recovery data for determination of 8 elements in animal feed by atomic absorption and inductively coupled argon plasma emission spectrometry

Element	Added, ppm	Recovered,[a] $\bar{x} \pm$ SD, ppm	Recovered,[a] $\bar{x} \pm$ SD, %
Arsenic	0.0	0.340 ± 0.036	—
	0.1	0.430 ± 0.023	91.0 ± 5.4
	0.2	0.510 ± 0.019	85.0 ± 3.7
	0.5	0.830 ± 0.034	98.0 ± 4.1
Cadmium	0.0	0.22 ± 0.01	—
	0.1	0.30 ± 0.01	80.0 ± 3.3
	0.2	0.40 ± 0.02	90.0 ± 5.0
	0.3	0.51 ± 0.02	96.7 ± 3.9
Calcium	0.0	11 700 ± 170	—
	500	12 100 ± 170	85.0 ± 2.9
	1000	12 500 ± 210	86.6 ± 3.2
	1500	13 300 ± 240	107.0 ± 3.5
Copper	0.0	11.8 ± 0.1	—
	2.0	13.7 ± 0.15	95.0 ± 2.1
	4.0	15.5 ± 0.4	92.5 ± 4.2
	6.0	17.3 ± 0.3	91.7 ± 3.4
Lead	0.0	0.9 ± 0.05	—
	0.5	1.3 ± 0.1	80.0 ± 7.7
	1.0	1.8 ± 0.1	90.0 ± 5.5
	1.5	2.3 ± 0.15	93.3 ± 6.5
Mercury	0.0	0.03 ± 0.005	—
	0.05	0.072 ± 0.005	84.0 ± 6.9
	0.1	0.135 ± 0.010	105.0 ± 7.4
	0.2	0.210 ± 0.020	91.3 ± 5.0
Selenium	0.0	0.310 ± 0.031	—
	0.1	0.405 ± 0.020	91.0 ± 4.9
	0.2	0.498 ± 0.026	93.0 ± 5.2
	0.5	0.815 ± 0.032	100.4 ± 3.9
Zinc	0.0	111 ± 1.4	—
	10.0	120 ± 1.0	90.0 ± 1.7
	20.0	132 ± 2.9	105.0 ± 3.6
	30.0	140 ± 1.7	96.7 ± 2.8

[a] Mean and standard deviation ($n - 1$) from triplicate assays.

Results of assays of animal feed fortified with 3 levels of each element are presented in Table 1. Recoveries of all elements at all levels were between 80 and 107%. Minimum detectable levels (based on twice background noise) were arsenic 0.025 ppm, cadmium 0.025 ppm, calcium 1 ppm, copper 0.5 ppm, lead 0.1 ppm, mercury 0.010 ppm, selenium 0.05 ppm, and zinc 0.5 ppm. Reagent blanks were used for zero concentrations.

Table 2 presents results for 3 methods: for arsenic determination by colorimetry with silver

Table 2. Comparison of results for arsenic and selenium determinations in animal feed by different methods

	As, mg/g ($\bar{x}$ ± SD)[a]		Se, mg/g ($\bar{x}$ ± SD)[a]	
Batch	Assay of hydrides[b]	Colorimetric[c,d]	Assay of hydrides[b]	SPF[c,e]
3555	320 ± 41	240 ± 9	270 ± 20	350 ± 50
3556	320 ± 60	230 ± 13	280 ± 33	290 ± 25
3557	340 ± 20	240 ± 13	300 ± 30	300 ± 21
3558	300 ± 30	240 ± 10	300 ± 30	260 ± 10
3559	360 ± 20	250 ± 9	280 ± 30	280 ± 16

[a] Mean and standard deviation ($n - 1$) are determined from triplicate assays corrected for % recovery and reagent blank.
[b] Method described in this paper for simultaneous determination of arsenic and selenium.
[c] Method formerly used for animal feed analyses at the National Center for Toxicological Research.
[d] Arsenic determination by colorimetry with silver diethyldithiocarbamate.
[e] Selenium determination by spectrophotofluorescence (SPF) with diaminonaphthalene.

diethyldithiocarbamate, as described by Hundley and Underwood (23); for selenium determination by spectrophotofluorescence with diaminonaphthalene (DAN) (24); and for simultaneous determination of arsenic and selenium by hydride generation with detection by AA. All results were corrected for the percent recoveries shown in Table 1. Compared with the assay of the hydrides, the colorimetric analyses for arsenic gave low results, whereas the results for selenium by spectrophotofluorescence agreed favorably. We feel that loss of arsenic occurred in the dry-ashing procedure which involves heating a 20 g feed sample at 550°C in the presence of magnesium oxide and magnesium nitrate. The feed preparation analyzed contains 10% fish meal for which arsenic concentrations of 2.6–19.1 ppm with a mean of 6 ppm have been reported (1). These data indicate that the higher values for arsenic are correct.

Typical recorder responses for arsenic and selenium in standards, animal feed, and animal feed fortified with 100 ppb of each element are presented in Figure 2. Figure 3 illustrates typical recorder responses for mercury in standards,

Figure 2. Typical recorder responses from AAS determinations of arsenic and selenium in standards, animal feed, and animal feed fortified with 100 ppb of each element.

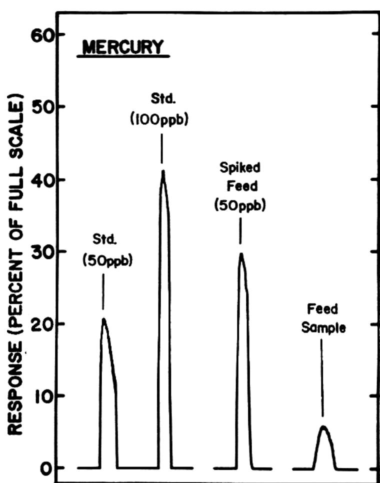

Figure 3. Typical recorder responses from AAS determinations of mercury in standards, animal feed, and animal feed fortified with 100 ppb mercury.

Table 3. Analysis of animal feed by atomic absorption (AA) and inductively coupled argon plasma (ICAP) emission spectrometry[a]

	Found by AA, μg/g					Found by ICAP, μg/g		
Batch	As	Cd	Pb	Hg	Se	Ca	Cu	Zn
3555	0.320	0.220	0.9	<0.025	0.270	13 600	13.6	99
3556	0.320	0.210	0.7	<0.025	0.280	13 200	13.0	98
3557	0.340	0.180	0.8	<0.025	0.300	13 700	13.4	87
3558	0.330	0.190	0.9	<0.025	0.300	11 200	11.1	88
3559	0.360	0.180	1.2	<0.025	0.280	11 700	11.8	123

[a] Each value is the average of duplicate determinations corrected for % recovery.

animal feed, and animal feed fortified with 100 ppb of the element.

Typical analyses of 5 batches of animal feed are presented in Table 3. The values reported meet the tentative NCTR requirements for animal feed as previously stated. The concentrations were corrected for the percent recoveries shown in Table 1.

Conclusions

The methods formerly used for animal feed analyses at the NCTR involved the ashing of 4 separate samples in a muffle furnance plus 2 wet digestions. Cadmium, calcium, lead, and zinc were assayed by AA, and arsenic and copper were determined colorimetrically. Selenium was determined by spectrophotofluorescence with DAN, and mercury was determined by a cold vapor method with a mercury meter. The methods included in this paper require a total of 3 digestions for all of the analyses. Simultaneous determinations include those for calcium, copper, and zinc by ICAP; cadmium and lead by flameless AA; and arsenic and selenium by AA in an argon-hydrogen flame. These methods have reduced the analysis time required to determine these 8 elements in animal feed by more than 30%.

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DRUGS IN FEEDS

High Performance Liquid Chromatographic Method for Pyrantel Tartrate in Swine Feeds and Supplements

JOHN T. GORAS
Pfizer Inc., Analytical Research Department, Pfizer Central Research, Groton, CT 06340

A new method for the determination of pyrantel tartrate in swine feed and supplements has been developed because the current official AOAC method is not applicable to feeds co-medicated with tylosin. The new method involves: (a) leaching of drug from feed with methanolic NaCl solution, (b) removal of interfering substances by ion pair liquid-liquid extraction and high performance liquid chromatography, and (c) quantitation of pyrantel tartrate by monitoring the ultraviolet absorption of the effluent stream at 313 nm. The method of standard addition is used to compensate for the effect of the feed matrix on drug recovery. No interference is encountered from tylosin, carbadox, lincomycin, non-drug components of feeds and supplements, or potential degradation products of pyrantel tartrate, i.e., *cis* isomer of pyrantel tartrate and (E)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide. Results for the assay of 3 lots each of feeds and supplements containing 0.0106 and 0.106% pyrantel tartrate, respectively, were within ±4% of label claim. Coefficients of variation ranged from 1.6 to 1.8% for feeds and from 1.9 to 3.9% for supplements.

Pyrantel tartrate (1,4,5,6-tetrahydro-1-methyl-2-(*trans*-2-(2-thienyl)vinyl)pyrimidine tartrate, Banminth®) is an effective anthelmintic agent that has been approved for veterinary use. In one application it is incorporated into swine feeds at a concentration of 0.0106% and into swine supplements at various higher levels, e.g., 0.106%. A spectrophotometric method for the determination of pyrantel tartrate in feeds and supplements was first reported by Lynch (1). A modified version of the Lynch method was collaboratively studied by Litchman (2, 3) and adopted official final action in 1980.

The option to use pyrantel tartrate in combination with an antibacterial has practical and economical advantages. The antibacterials carbadox and tylosin have been approved by the Food and Drug Administration for use in combination with pyrantel tartrate in feed. Regulatory approval for the combination of pyrantel tartrate and lincomycin has been requested.

Neither carbadox nor lincomycin interferes in the determination for pyrantel tartrate in feed by the official AOAC method. However, tylosin does interfere by responding as pyrantel tartrate in the final spectrophotometric measurement. Consequently, a new method has been developed for pyrantel tartrate in feeds containing either tylosin, carbadox, or lincomycin. This method involves the following sequence of steps: (a) pyrantel tartrate is leached from feed or supplement with methanolic sodium chloride solution, (b) interfering substances are removed by a combination of ion pair liquid-liquid extraction and high performance liquid chromatography (HPLC) on a silica column, and (c) pyrantel tartrate is measured in the effluent stream by monitoring of ultraviolet absorption at 313 nm. As in the official AOAC method, the new procedure requires the use of the method of standard addition to avoid a low bias due to the effect of some feed matrices on the recovery of pyrantel tartrate. No interference is encountered from the *cis* isomer of pyrantel or (E)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide. These potential degradation products of pyrantel may be generated by photodecomposition and alkaline decomposition, respectively.

METHOD

(Caution: Solutions of pyrantel tartrate are light-sensitive. Protect standard solutions and extracts from direct sunlight or artificial light.)

Reagents

(a) *Methanolic NaCl solution.*—Dissolve 100 g NaCl in 1 L water. Add 1 L methanol and mix. Prepare fresh daily.

(b) *KI solution.*—Dissolve 110 g KI in enough water to make 200 mL. Prepare fresh daily.

(c) *NaOH solution.*—0.1N. Dissolve 4 g NaOH in enough water to make 1 L. Prepare fresh daily.

(d) *Mobile phase.*—Acetonitrile–water–acetic acid–diethylamine (94 + 2.5 + 2.5 + 1). Filter under vacuum through 5 μm Teflon® filter (Millipore Mitex No. LSWP04700, Millipore

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Corp., Bedford, MA 01730). Degas by stirring 10 min under vacuum. Prepare fresh daily.

(e) *Pyrantel tartrate standard solutions.*—Prepare fresh daily using pyrantel tartrate reference standard (available from Pfizer, Inc., Lee's Summit, MO 64063). Use an ultrasonic bath to hasten dissolution. Standard solution A for 0.0106% pyrantel tartrate feeds: Dissolve 21.2 mg reference standard in enough methanolic NaCl solution to make 100.0 mL. Standard solution B for 0.106% pyrantel tartrate supplements: Dissolve 26.5 mg reference standard in enough methanolic NaCl solution to make 25.0 mL.

Apparatus

(a) *High performance liquid chromatograph.*—Model 98750, 3000 psi pump, and Model 440 spectrophotometric detector (Waters Associates, Milford, MA 01757), or equivalent, operated at flow rate of 1.6 mL/min and detector wavelength at 313 nm, 0.2 AUFS. Model 385 recorder (Linear Instruments Corp., Irvine, CA 92714), or equivalent, operated at 5 mV (sample) and 10 mV (spiked sample) and 12 cm/h chart speed. Model CV-6-UHPa-N60 injection valve with 25 μL injection loop (Valco Instruments Co., Inc., Houston, TX 77055), or equivalent.

(b) *Chromatographic column.*—4.6 mm id × 25 cm Zorbax® Sil, Part No. 850952701 (DuPont Co., Wilmington, DE 19898) with Model 84550 guard column containing 400 mg Corasil II (Waters Associates).

(c) *Ultrasonic bath.*—Model 52, 2 gal., 200 watts (Branson Cleaning Equipment Co., Shelton, CT 06484), or equivalent.

(d) *Rotary evaporator.*—Model PFE-1BN (Buchler Instruments, Inc., Fort Lee, NJ 07024), or equivalent, with vacuum pump, solvent trap, and 45°C water bath.

Preparation of Samples

Grind about 40 g feed or supplement 30 s in a high-speed blender. Weigh duplicate portions of ground sample into 125 mL Erlenmeyer flasks: 10.00 g for 0.0106% pyrantel tartrate and 5.00 g for 0.106% pyrantel tartrate. Add 100 mL methanolic NaCl solution to one flask. Prepare spiked sample by adding 95 mL methanolic NaCl solution and 5.00 mL standard solution A (for 0.0106% pyrantel tartrate) or B (for 0.106% pyrantel tartrate) to other flask. Insert polyethylene stopper, place in ultrasonic bath 1 h, and then cool to room temperature (bath temperature increases to 55–75°C). Using three 20 mL washings with methanolic NaCl solution, quantitatively transfer mixture to Buchner funnel containing prefilter pad (Millipore AP2504700) covered with 1.5 g glass wool pad. Collect filtrate under vacuum in 200 mL volumetric flask. Let filtrate reach room temperature, fill to mark with methanolic NaCl solution, and mix.

Pipet 25.0 mL filtrate for 0.0106% pyrantel tartrate or 5.00 mL filtrate plus 20 mL water for 0.106% pyrantel tartrate into 125 mL separatory funnel containing 10 mL KI solution. Swirl gently to mix. Add 50 mL $CHCl_3$ to the separatory funnel and shake vigorously 10 s. Drain lower $CHCl_3$ layer into 250 mL separatory funnel containing 25 mL 0.1N NaOH solution. Re-extract aqueous KI layer with second 50 mL portion of $CHCl_3$. After separation, drain $CHCl_3$ layer into separatory funnel containing first $CHCl_3$ extract and NaOH solution. Shake separatory funnel vigorously 10 s and let layers separate. Drain lower $CHCl_3$ layer into 500 mL round-bottom flask and evaporate just to dryness with rotary evaporator. Reconstitute residue in flask with 10.0 mL mobile phase solution. Stopper tightly until ready to use.

Determination

Inject sample and spiked sample solutions into HPLC system. Determine response of sample (R) and sample spiked with appropriate standard (R') from height or area of pyrantel peak (retention time = ca 7.3 min). If manual syringe injection is used, make multiple injections of 25 μL in sequence of $R'RRR'$ and calculate average values for R and R', respectively. Calculate pyrantel tartrate content of feed from following equation:

% Pyrantel tartrate = $[R \times$ (g pyrantel tartrate/mL std soln) $\times$ 5 mL std soln $\times$ 100$]/[(nR' - R) \times$ g feed]

Note: n = recorder input voltage for sample spiked with standard/recorder input voltage for sample.

Results and Discussion

When pyrantel tartrate standard solution is taken through the assay procedure in the absence of feed, a single sharp peak with a retention time of about 7.3 min is obtained (see Figure 1). Assay conditions provide 50% full scale deflection (5 mV recorder input) for 0.32 μg pyrantel tartrate standard. Evidence that pyrantel carries through the assay procedure and elutes from the HPLC column as the intact drug was obtained by collecting and identifying the fraction associated with this peak. The isolated fraction exhibited

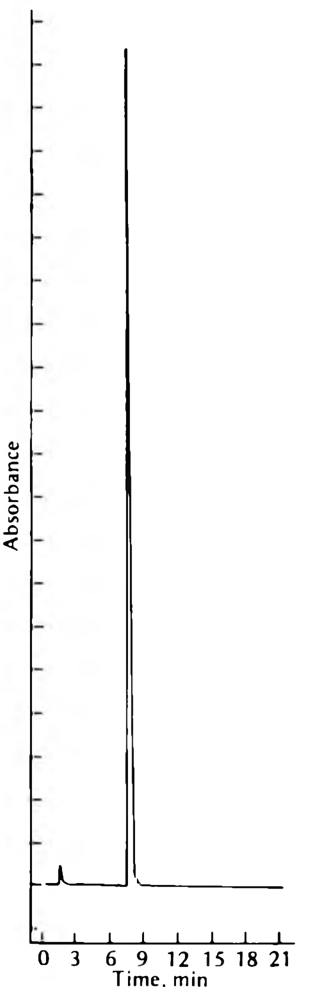

Figure 1. Liquid chromatogram for pyrantel tartrate standard solution carried through assay procedure in absence of feed.

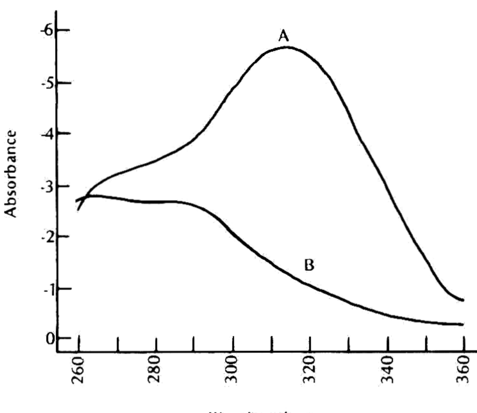

Figure 2. Ultraviolet absorption spectra for isolated HPLC fraction before irradiation (A) and after irradiation (B) with intense long wavelength ultraviolet light.

an ultraviolet absorption spectrum that is characteristic of pyrantel with an absorption maximum at about 311 nm (see curve A, Figure 2) (1). After the same fraction was irradiated with intense long wavelength ultraviolet light, a different spectrum with absorption maxima at about 270 and 285 nm was obtained (see curve B, Figure 2). The latter spectrum is characteristic of the *cis* isomer of pyrantel, which is produced by photolytic degradation of the drug (1, 4).

Absence of interference from feed and supplement components was demonstrated by applying the method for 0.0106% pyrantel tartrate to 11 different commercial type formulations that were devoid of medication (see Table 1). None of these formulations exhibited components eluting at or near the retention time for pyrantel tartrate. A typical chromatogram, which was obtained for formula C in Table 1, is shown in Figure 3.

Absence of interference from the 3 antibacterials in question and the 2 potential degradation products of pyrantel was demonstrated by applying the method for 0.0106% pyrantel tartrate to a specially prepared blend made with feed C. The blend, which was devoid of pyrantel tartrate, contained 0.00551% carbadox and 0.011% each of lincomycin, tylosin, *cis* isomer of pyrantel tartrate, and (E)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide. The chromatogram obtained for this blend showed no components eluting at the retention time for pyrantel tartrate (see Figure 4). No peak was found for either carbadox or lincomycin. The chromatogram obtained for a sample of the same blend to which 0.0106% pyrantel tartrate was added shows complete resolution of pyrantel tartrate from all 5 potential contaminants (see Figure 5). Separation factors were calculated relative to the pyrantel tartrate peak. The values for tylosin, *cis* isomer of pyrantel tartrate, and

Table 1. Unmedicated formulations tested for assay interference

Formula	Type of formulation
A, B	18% protein swine starter
C	16% protein swine weaner
D, E, F	16% protein swine grower
G	14% protein swine developer
H, I	13% protein swine finisher
J, K	swine protein supplement

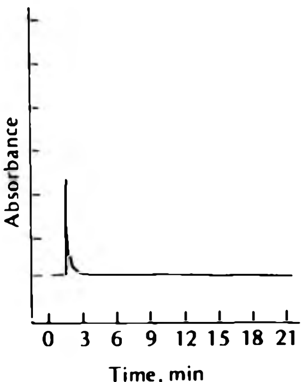

Figure 3. Liquid chromatogram for unmedicated feed C assayed by procedure for 0.0106% pyrantel tartrate.

(E)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide were 0.15, 1.40, and 2.95, respectively. Interference from sulfas was not checked, because such a combination is not approved.

The method of standard addition is used in the official AOAC method to compensate for a low bias due to feed matrix effect, an analytical phenomenon which affects recovery of pyrantel tartrate but has no relationship to bioavailability or efficacy. The feed matrix has a similar effect on the recovery of pyrantel tartrate in the proposed HPLC method. This was demonstrated by comparing fractional recovery values obtained with and without feed present. The fractional recovery was calculated from the response (i.e., peak height/weight of pyrantel tartrate) of a pyrantal tartrate standard solution that was carried through the assay procedure divided by the

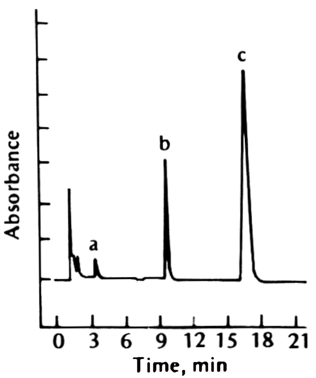

Figure 4. Liquid chromatogram for feed C containing 0.00551% carbadox and 0.011% each of lincomycin, tylosin (peak a), *cis* isomer of pyrantel tartrate (peak b), and (E)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide (peak c). Sample assayed by procedure for 0.0106% pyrantel tartrate.

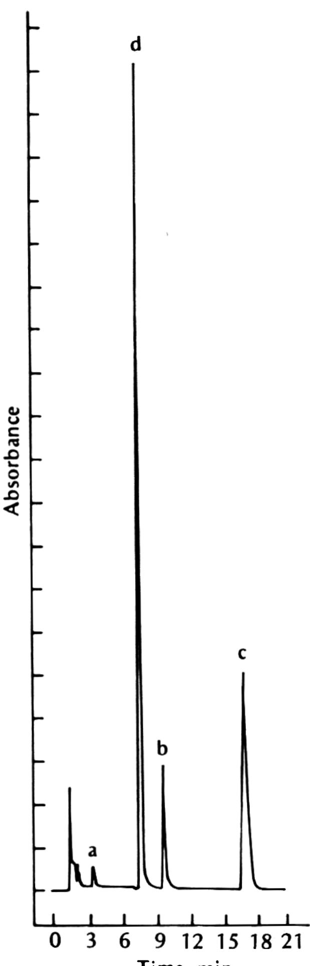

Figure 5. Liquid chromatogram for same sample described in Figure 4 with 0.0106% pyrantel tartrate added (peak d).

response of a pyrantel tartrate standard solution that was diluted by the same factor with mobile phase and directly injected into the HPLC. The fractional recovery in the absence of feed was high and reproducible, i.e., average ± standard deviation for 51 determinations = 0.947 ± 0.014. When the fractional recovery was determined in the presence of 16 different formulations that were devoid of pyrantel tartrate, several low values were found: Unmedicated commercial type feed rations B, E, F, G, H, and I gave 0.875, 0.935, 0.937, 0.918, 0.903, and 0.941, respectively; commercial rations containing carbadox, L, M, N, O, P, Q, R, S, T, and U, gave 0.926, 0.882, 0.447, 0.937, 0.897, 0.712, 0.898, 0.920, 0.904, and 0.931, respectively. These data demonstrate that assay results for some feeds would have a significant

Table 2. Reproducibility of assay of pilot scale lots of feeds and supplements

	0.0106% Pyrantel tartrate[a]			0.106% Pyrantel tartrate		
Detn	Lot 1	Lot 2[b]	Lot 3[b]	Lot 4[c]	Lot 5[d]	Lot 6[e]
1	0.0107	0.0108	0.0114	0.107	0.101	0.105
2	0.0107	0.0109	0.0112	0.108	0.105	0.105
3	0.0109	0.0109	0.0108	0.108	0.104	0.105
4	0.0108	0.0107	0.0111	0.105	0.098	0.106
5	0.0111	0.0109	0.0111	0.106	0.106	0.107
6	0.0108	0.0107	0.0114	0.111	0.103	0.110
7	0.0111	0.0108	0.0109	—	0.101	—
8	0.0112	0.0109	0.0109	—	0.095	—
9	0.0108	0.0112	0.0107	—	0.107	—
10	0.0108	0.0112	0.0111	—	0.097	—
Av.	0.0109	0.0109	0.0111	0.108	0.102	0.106
SD	0.0002	0.0002	0.0002	0.002	0.004	0.002
CV, %	1.6	1.6	1.8	1.9	3.9	1.9

[a] Pelleted feeds made from formulation A (Table 1).
[b] Feed co-medicated with 0.011% tylosin.
[c] Meal made from formulation J (Table 1), co-medicated with 0.055% tylosin.
[d] Meal made from formulation K (Table 1), co-medicated with 0.110% lincomycin.
[e] Meal made from formulation K (Table 1).

low bias without appropriate compensation for the effect of the matrix. Review of label ingredients for the various feeds does not provide any clues as to what component(s) may be responsible for the matrix effects.

Formulations N and Q, which exhibited the lowest fractional recovery values above, were used to demonstrate that the method of standard addition compensates for the effect of feed on the recovery of drug. Pyrantel tartrate in methanolic sodium chloride solution was added to duplicate samples of N and Q to simulate analytical samples of feeds containing 0.0106% pyrantel tartrate. One sample from each pair was spiked with 5.00 mL pyrantel tartrate standard solution A. Additional methanolic sodium chloride solution was added to each sample to bring the total volume of each solution to 100 mL. The samples were then treated as described for 0.0106% pyrantel tartrate, starting with "Insert polyethylene stopper. . . ." The results indicated that 96 and 98% of the pyrantel tartrate added to formulations N and Q, respectively, was found by using the method of standard addition.

A similar experiment was performed to demonstrate the linearity of the method over the range of the permitted analytical variation for pyrantel tartrate, i.e., ± 25% of label claim. Samples simulating feeds containing 0.0079% (25% below label claim), 0.0106% (label claim), and 0.0132% (25% above label claim) pyrantel tartrate were prepared and analyzed as described above. Unmedicated feed formulation A (Table 1) was used in this experiment. The results indicated that at each level, the amount of pyrantel tartrate found was in excellent agreement with the amount added (added: 0.0079, 0.0106, 0.0132%; found: 0.0082, 0.0106, 0.0138%).

Three lots of complete swine feeds and 3 lots of supplements containing 0.0106 and 0.106% pyrantel tartrate, respectively, were used to evaluate reproducibility of the method. The 6 lots, which weighed about 100 lb each, were manufactured from commercial premixes and formulas A, J, or K. Three lots were co-medicated with tylosin and one lot was co-medicated with lincomycin. Results for 6 to 10 determinations on each lot demonstrated satisfactory reproducibility (see Table 2). The coefficients of variation ranged from 1.6 to 1.8% for feeds and 1.9 to 3.9% for supplements. In all cases, average potency values were in good agreement with label claim (i.e., 96–104% of label claim).

In summary, the HPLC method described above provides a reliable means for determining pyrantel tartrate in feeds and supplements that, because of the presence of tylosin, cannot be assayed by the official AOAC method. Data relative to specificity, reproducibility and accuracy indicate that the method is a potential replacement for the current official AOAC method. With appropriate modification of sample weight, volume of spike solution, and dilution factor, the HPLC method can be applied to supplements at other potency levels.

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METALS AND OTHER ELEMENTS

Atomic Absorption Spectrometric Determination of Tin in Canned Foods Using Nitric Acid-Hydrochloric Acid Digestion and Nitrous Oxide-Acetylene Flame

ROBERT W. DABEKA and ARTHUR D. MCKENZIE
Health and Welfare Canada, Food Directorate, Food Research Division, Health Protection Branch, Ottawa, Ontario, Canada K1A OL2

Foods (5-40 g) are digested sequentially with HNO_3 and HCl, diluted to 100 mL, and filtered, and tin is determined by atomic absorption using an N_2O-C_2H_2 flame. Synthetic standards in 10% HCl are used. A positive interference by potassium is compensated by adding 100 μg potassium/mL to both standards and samples. Recoveries for 17 foods spiked with tin at levels between 40 and 500 μg/g ranged from 85 to 110% with a mean of 101%. Results agreed well with a solvent extraction atomic absorption method and a direct X-ray fluorescence method.

Regulatory and monitoring agencies usually require the determination of tin in canned foods at levels between 5 and 500 ppm. Although flame atomic absorption spectrometry is rapid and appears to be the most convenient instrumentation for determining tin at such levels, it is still beset by problems, among which are interferences due to sulfuric and perchloric acids, organic constituents, and various cations. As a result, well studied general methods still rely on the complete destruction of organic matter (1-3), and standard additions (2) or separation steps (3) must be used. These often increase analytical time, increase hazards associated with use of strong oxidants such as perchloric acid for complete digestions, and preclude convenient use of the methods for routine applications.

In this paper a convenient routine method, which has the advantages of a less rigorous digestion, and the use of synthetic standards rather than standard additions is described.

METHODS

Apparatus

(a) *Atomic absorption spectrometer.*—Equipped with N_2O-C_2H_2 burner and simultaneous background correction (Varian AA-775, Varian Associates of Canada Ltd, Ottawa, Ontario).

(b) *X-ray fluorescence (XRF) spectrometer.*—Wavelength dispersive. Philips universal vacuum X-ray spectrometer with W target, LiF crystal ($2d$ = 4028 A), and NaI(T1) scintillation detector, Model PW-1410 (Philips Electronic Equipment Ltd, Toronto, Ontario).

Reagents

(a) All reagents were reagent grade, except high-purity HCl which was prepared by subboiling distillation and used only for XRF method. Deionized water was used throughout study.

(b) *Tin stock solution.*—1000 μg/mL. Prepare according to ref. 2.

(c) *Potassium stock solution.*—10 000 μg/mL. Dissolve 19.1 g KI in water and dilute to 1 L with water.

Preparation of Samples

If necessary for homogeneity, dilute samples (1 + 1 by weight) with water and shake or blend. Accurately weigh food into 250 mL Erlenmeyer flask: 30-40 g for juices or drinks, 20 g for foods containing 50-75% water, and 5-10 g for solids or semisolids. Limit fat or oil content to 2-4 g. Dry food in oven at ca 120-140°C. (Charring, which may occur during overnight drying, is permissible.)

Add 30 mL HNO_3 to flask and heat gently to initiate digestion, avoiding excessive frothing. When frothing has subsided, heat at moderate temperature (gentle boil) until 3-6 mL digest remains. Initial stages of sample drying on bottom of the flask will not affect recovery; however, charring or spattering of sample should be avoided. Remove flasks from heat.

Starting with next steps, carry 2 sample blanks through entire procedure. Add 25 mL HCl to flask, and gently boil solution until 10-15 mL digest remains. Remove from heat. Complete HNO_3-HCl digestion requires ca 4 h.

Pipet 1.0 mL potassium stock solution into 100 mL volumetric flasks for samples and blanks. Transfer warm digest to volumetric flask, thor-

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oughly washing Erlenmeyer flask twice with water. Transfer washings to volumetric flask. Let solution cool to room temperature and dilute to volume with water. Fat floating on top should not be considered part of volume. Mix well and filter ca 50 mL through dry paper (Whatman No. 1) and funnel into clean, dry bottle.

Standard Preparation

Into six 100 mL volumetric flasks, pipet 1.0 mL potassium stock solution; then add ca 10 mL HCl. Pipet 0, 5, 10, 15, 20, and 25 mL tin stock solution (1000 μg/mL) into flasks and dilute to volume with water.

Determinations

Set up and optimize atomic absorption spectrophotometer with simultaneous background correction and N_2O–C_2H_2 flame according manufacturer's recommendations. Use 233.5 nm wavelength. Aspirate sample, then standard with approximately same absorbance. Calculate concentration of tin in sample, using absorbance ratio and allowing for sample weight, dilutions, and sample and standard blanks.

Extraction Comparative Method

Samples were digested with HNO_3 and H_2SO_4, and tin was extracted with KI and toluene according to Engberg (3). Tin was then stripped into 20 mL solution containing HCl (10%) and H_2O_2 (2%), and determined by atomic absorption as above.

X-Ray Fluorescence Comparative Method

Each sample was divided into four 4 g portions, and 4 mL each of 10% high-purity HCl, containing <1 ng tin/mL, and 3 standards, containing 100, 200, and 400 μg tin/mL 10% HCl were added. Mixtures were blended, put into single liquid sample holder, and counted for 1 min at 13.60°, 14.00° and 14.40° (2θ). Corrections for sample background at 13.60° and 14.40°, and for background curvature at 13.60°, 14.00°, and 14.40° for cell containing 10% high-purity HCl were applied, and tin concentrations were calculated using method of additions.

Results and Discussion

Sample Digestion.—To avoid the time and attention necessary for complete destruction of organic matter, several authors have described the direct partial digestion of foods with HCl, followed by filtration of charred matter (4–7). This technique, coupled with determination by atomic absorption (air–C_2H_2 and N_2O–C_2H_2 flames), has been extensively applied by the 5 field laboratories of the Canadian Health Protection Branch; however, a collaborative study, conducted with these laboratories to check the method, revealed it to be insufficiently rugged and well defined (R. W. Dabeka & M. Mankotia, unpublished data); particular problems were encountered with tomato paste. For instance, using an N_2O–C_2H_2 flame, recoveries of a 226 μg/g spike from tomato paste varied from 64 to 95%.

Closer investigation of the method in our laboratory revealed that, after digestion, a large amount of organic material remained in solution and, for N_2O–C_2H_2 stainless steel burners, could cause rapid burner clogging. For instance, digestion of 40 g tomato paste with HCl, dilution to 100 mL, and filtration yielded a solution that gave a 6% residue after drying at 120°C. The percent residue was directly proportional to the sample size for sizes ranging from 2.5 to 40 g. Application of the sequential HNO_3–HCl digestion lowered the organic residue by a factor of 3, significantly reducing burner clogging.

Precipitation of metastannic acid in the presence of boiling HNO_3 resulted in 50% losses of tin standards carried through the complete digestion; however, losses were not observed when samples were digested. This appears to be due to the presence of unoxidized organic matter.

Blanks for the samples were started from the HCl digestion step because tin levels in reagent grade HNO_3 are usually in the ng/g range. In spite of the validity of this approximation, it is noteworthy that the contribution of HNO_3 to the blanks cannot be truly evaluated for this method because of precipitation of metastannic acid during HNO_3 digestion.

Interferences.—During initial development of the method, recoveries of tin spikes averaged 109% and a sample-dependent positive matrix effect was observed. The effect was usually constant for sample sizes ranging from 1 to 40 g; therefore, it did not appear to be caused by undigested organic matter. When the sodium, potassium, and calcium composition of the standards matched that of the samples, recoveries approximated 100%. Additional studies defined potassium as the primary enhancement factor, and for standard solutions containing 50–400 μg potassium/mL, absorbances increased by 6% and remained relatively constant over this concentration range. A similar enhancement (10%) by potassium was observed by Wehrer et al. (7) in an air–C_2H_2 flame; however, for N_2O–C_2H_2 flames, cationic interferences have not been reported (8).

Table 1. Recovery of tin from foods

Sample	Sample size, g	Found, μg/g ± SD[a]	Spike level, μg/g[b]	Recovery, % ± SD
Canned meat	10	11.5 ± 0.7		
	10		100	104 ± 9
	10		250	103 ± 4
Canned peaches	20	31 ± 2		
	20		50	98 ± 4
	20		250	100.7 ± 0.1
Canned spaghetti in tomato sauce	20	62 ± 1		
	20		50	85.0 ± 0.0
	20		250	104.8 ± 0.2
Grapefruit juice unsweetened	40	49.0 ± 0.4		
	40		50	106 ± 3
	40		250	101 ± 2
Evaporated milk	40	22.0 ± 0.3		
	40[c]		50	101.5
	40		250	103 ± 3
Canned tomatoes	20[c]	2		
	20		50	97 ± 5
	20		250	99 ± 2
Canned fish	10	<3		
	10		100	100.5 ± 0.7
	10		250	106.2 ± 0.3
Canned asparagus	20	144 ± 2		
	20		50	110 ± 2
	20		250	98 ± 8
Tomato juice	50	13.3 ± 0.1		
	50		50	99 ± 6
	50		250	100 ± 2
Vegetable cocktail juice	40	11.3 ± 0.8		
	40		50	105.5 ± 0.7
	40		250	99.9 ± 0.8
Canned pineapple	40	106 ± 5		
	40		50	95 ± 8
	40		250	97.3 ± 0.6
Canned tomato paste	20	17 ± 1		
	20		100	99 ± 2
	20		500	102.7 ± 0.8
Tomato ketchup	30	<2		
	30		100	104 ± 4
	30		330	101.4 ± 0.4
Apple juice	50	<1		
	50		50	104 ± 0.6
	50		200	103.4 ± 0.4
Canned pork and beans in tomato sauce	20	3.3 ± 0.4		
	20		100	105 ± 4
	20		250	99 ± 3
Orange juice unsweetened	50	60 ± 3		
	50		40	99 ± 3
	50		200	101 ± 3
Canned soft drink beverage	40	<1		
	40		60	101 ± 3
	40		250	97.6 ± 0.8

[a] Two replicates, except where noted.
[b] Canadian regulatory limits for tin in foods are set at 250 ppm.
[c] Single analysis.

The plateau-type enhancement by potassium was applied to the procedure, and potassium (100 μg/mL in the final solution) was subsequently added to all standards and samples.

Recoveries improved substantially and relationships between macroelement composition, flame stoichiometry, and the presence of organic matter were not considered further.

Hydrochloric acid, in the concentration range 0.6–3.0M, and traces of HNO_3, up to 4% in the final solution, did not affect tin recovery.

Accuracy.—The accuracy of the method was

Table 2. Comparison of proposed method for tin determination with other techniques

Sample	Sn found (µg/g ± SD)		
	This method[a]	Solvent extn[a]	XRF
Pineapple juice	94 ± 6	92 ± 5	84
Evaporated milk	17 ± 3	11.9 ± 0.8	20
Canned asparagus	146 ± 1	135 ± 17	142
Orange juice	49.9 ± 0.9	48.1 ± 0.6	166[b]
Canned spaghetti	75 ± 4	80 ± 10	70
Tomato paste	140 ± 2	137 ± 2	164

[a] Three replicates per food.

[b] Sample had been stored in its can for 2 weeks after the first 2 methods were used and the high level may be due to leaching of tin.

tested by recovery studies and by comparing the method with solvent extraction followed by flame atomic absorption and with X-ray fluorescence of undigested foods by using standard additions.

Recovery of tin spiked at 2 levels in 17 foods ranged from 85 to 110%, with a mean of 101% (Table 1). Recovery at lower spiking levels (40–100 µg/g) was more erratic (5.6% RSD) due to measurement imprecision, but improved considerably (2.5% RSD) for the higher spikes (200–500 µg/g).

Results obtained by the present method and those obtained using solvent extraction atomic absorption and XRF methods compared favorably (Table 2).

Conclusions

By using an N_2O-C_2H_2 flame rather than any of the cooler flames, interferences due to organic matter (9) and various cations (10) are reduced significantly. The potassium interference, still present in this flame, is compensated by addition of potassium to standards and samples. By using a sequential HNO_3-HCl digestion, organic matter, which can cause rapid clogging of the burner head, is reduced to a manageable level. Interference in the flame caused by sulfuric acid, frequently used in complete digestion methods, is avoided.

As a result of the above approach, the method is lengthier than direct HCl digestion methods, but is more reliable. The digestion step for the method does not require as much analytical attention as do existing methods in which complete destruction of organic matter is effected and, unlike such methods, the method does not require separation steps (1, 3) or the use of standard additions (2) to compensate for interferences. Thus, the method is suitable for routine determinations. Collaborative study of the method is being planned.

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Simple Paper and Micro Thin Layer Chromatographic Method for Separation and Detection of Mercuric Chloride, Copper Sulfate, Cadmium Sulfate, and Silver Nitrate in Fresh Water

YALAVARTHY PRAMEELA DEVI and NANGUNERI V. NANDA KUMAR
Sri Venkateswara University, Department of Zoology, Tirupati 517502, India

Simple concentration techniques are described for detecting heavy metals in water. Copper sulfate and cadmium sulfate are precipitated with alkaline phosphate buffer (pH 8.2); mercuric chloride and silver nitrate are concentrated through evaporation. Compounds redissolved in citric acid are suitable for paper and micro thin layer chromatographic-enzymatic separation by a 0.1% NaCl solvent system. The heavy metal compounds can be detected by horse liver acetone powder succinate dehydrogenase inhibition, using 2-(4-iodophenyl-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)-sodium succinate-*N*-methyl phenazonium methosulfate mixture as the chromogenic reagent. Microgram amounts of Cu, Ag, Hg, and Cd can be separated and identified by using this method.

Enzymatic methods using paper and micro thin layer chromatography for detection and determination of heavy metals like $HgCl_2$, $CuSO_4$, $CdSO_4$, and $AgNO_3$ were reported previously (1). A portable method using bio-detector strips was also reported for detection of these compounds (2). The present paper describes precipitation and evaporation techniques to concentrate the samples and thereby improve sensitivity. An improved solvent system is also developed for better separation. The concentration methods described are different from the conventional, tedious, and laborious methods (3-5) usually employed for atomic absorption spectroscopy (6-8). The detection method is simple and sensitive compared with nonenzymatic chromatographic methods (9-11). The applicability of enzymatic methods for screening, concentration, and direct detection from fresh water samples is described and discussed.

METHOD

Apparatus and Reagents

(a) *Mercuric chloride, copper sulfate, and silver nitrate.*— Prepare standards (British Drug Houses (BDH), India) in both distilled and fresh water at 2 mg/100 mL.

(b) *Cadmium sulfate.*—Prepare standard (BDH) in both distilled and fresh water at 5 mg/100 mL.

(c) *Citric acid.*—Prepare 2% citric acid (E. Merck, Darmstadt) in water.

(d) *Sodium phosphate buffers.*—pH 7.2 (0.2M) and pH 8.2 (0.4M).

(e) *2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)-sodium succinate-N-methyl phenazonium methosulfate (PMS) mixture.*—Prepare INT (BDH, India) 0.4% in pH 7.2 phosphate buffer, sodium succinate (BDH, Poole, UK) 2.5% in pH 7.2 phosphate buffer, and PMS (BDH, Poole, UK) 0.1% in pH 7.2 phosphate buffer. Mix 10 + 10 + 2.

(f) *Enzyme.*—Prepare 2% homogenate of horse liver acetone powder (Sigma Chemical Co., St. Louis, MO 63178) in ice cold pH 7.2 phosphate buffer as described earlier (1) and use immediately.

(g) *Silica gel G.*—BDH, India.

(h) *Filter paper.*—Whatman No. 3; cut 7.5 × 2.5 cm strips.

(i) *Sodium chloride.*—0.1, 0.5, and 1%. Dissolve 100, 500, and 1000 mg NaCl/100 mL water.

Concentration by Precipitation

Separately prepare fresh water standards containing 1 mg $CuSO_4$ and 5 mg $CdSO_4$/10 mL water. Add pH 8.2 sodium phosphate buffer (0.4M) in small aliquots until precipitation occurs; 10 mL standard requires about 1 mL pH 8.2 sodium phosphate buffer. About 1.0 mL buffer may be added in excess. Centrifuge 15 min at 2500 rpm. Decant supernate. Add small volumes of 2% citric acid until residue dissolves. For $CuSO_4$, add 10 volumes of pH 7.2 sodium phosphate buffer for each volume of citric acid required. For $CdSO_4$, add 10 volumes of distilled water for each volume of citric acid (buffer causes precipitation). Solutions are ready for chromatographic analysis.

Concentration by Evaporation

Add 2 mL 2% citric acid to aqueous standards. Evaporate aqueous standards (distilled and fresh water) containing 2 mg $CuSO_4$ and 5 mg $CdSO_4$/100 mL in Petri dishes in 80°C hot air

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oven to 10 mL. Do *not* evaporate to dryness. Dilute to 20 mL with pH 7.2 sodium phosphate buffer. If precipitate forms, add small amounts of 2% citric acid to redissolve. Solutions are ready for chromatographic analysis.

Add 2 mL 2% citric acid and evaporate distilled water and fresh water standards containing 5 mg $HgCl_2$/100 mL to 10 mL; dilute solution to 20 mL with pH 7.2 buffer for chromatographic analysis as described for $CuSO_4$.

Evaporate distilled water standards containing 2 mg $AgNO_3$/100 mL with 2 mL 2% citric acid to 10 mL and dilute with distilled water to 20 mL. Evaporate fresh water standards containing 2 mg $AgNO_3$/100 mL with 2 mL 2% citric acid to 10 mL. The distilled water standard is ready for chromatographic analysis; no precipitation occurs. However, $AgNO_3$ and $CdSO_4$ require buffer treatment on paper or thin layer chromatographic (TLC) plate to neutralize acidity. For $AgCl_2$ insoluble precipitate formed in fresh water, decant supernate after centrifugation at 2500 rpm. Mix $AgCl_2$ precipitate uniformly in pH 7.2 sodium phosphate buffer and apply on paper or TLC plate.

Screening of Heavy Metal by Detector Strips

Use detector strips (2), which respond at nanogram amounts, to confirm presence of heavy metals in prepared samples. Proceed with chromatographic separation and identification.

Paper Chromatography

Apply 20 μL prepared $CuSO_4$, $HgCl_2$, $CdSO_4$, and $AgNO_3$ solutions on separate Whatman No. 3 filter paper strips 2 cm above base, with graduated micro capillary. Blow hot air with hair dryer. Minimize spot spreading while applying by frequently drying with hair dryer. Place strips in 0.1% NaCl solvent. Develop paper strips 7 cm by ascending chromatography by hanging strips in glass jar (12 × 4 cm) (1). Remove strips, air-dry, and then spray sodium phosphate buffer 2 or 3 times to neutralize citric acid effect for $CdSO_4$ and $AgNO_3$ samples only ($CdSO_4$ is prepared with distilled water). Air-dry after each buffer treatment to avoid leaching. Observe white spots of heavy metal compounds against pink background following enzyme-chromatogenic treatment (1). Calculate R_f values.

Micro Thin Layer Chromatography

Coat 7.5 × 2.5 cm glass slide with 450 μm layer of silica gel G in water slurry (22 g in 50 mL distilled water) by dipping micro slides. Air-dry plates and activate 1 h in 110°C air oven. Store plates in desiccator before use. Apply 20 μL $CuSO_4$, $CdSO_4$, $HgCl_2$, and $AgNO_3$ solutions on separate micro TLC plates 1.5 cm above base, with graduated micro capillary; blow hot air with hair dryer. Minimize spreading of applied spot by frequent drying with hair dryer. Place micro slides in 0.1% NaCl solvent system. Develop 7 cm by ascending chromatography by placing micro slides in 12 × 4 cm glass jar. Remove slides and blow hot air gently with hair dryer; spray horse liver acetone powder enzyme solution. Follow slightly modified and simplified method described below without placing slides in moist incubation chamber described earlier (1).

Place micro slides on glass plate after spraying enzyme, and let stand at room temperature (28°C) 4 min to allow inhibition of enzyme by heavy metal compounds. Micro slides should not dry. Avoid leaching by spraying excess enzyme solution or buffer if needed. Spray INT-sodium succinate-PMS (10 + 10 + 2) mixture as fine mist (1). Do not over-spray, which causes leaching. Place micro TLC plates in hot air oven (80°C) along with glass plate. Heavy metal inhibition of succinate dehydrogenase appears as white spot in pink farmazon (1, 2) background within 10-15 min. Lower limit of detection is 1 μg, except 5 μg $CdSO_4$.

For field analysis, follow methods described earlier (1, 2).

Results and Discussion

In the present method, $CuSO_4$ and $CdSO_4$ are concentrated through precipitation by adding pH 8.2 sodium phosphate buffer. Phosphate buffer precipitation is recommended for enzymatic methods of analysis instead of acid and alkaline treatments (5) which denature enzyme (12). The pH 8.2 sodium phosphate buffer does not affect enzyme activity; the succinate dehydrogenase itself exhibits optimal activity at pH 8.4. This single step precipitation is preferable to precipitation techniques employing thionalide, which are lengthy, tedious, and complex and each step increases the contamination of the sample with foreign material (13, 14). Precipitation was complete as confirmed by the absence of inhibitory factor in the supernate as tested by the present TLC enzymatic method.

Evaporation of aqueous standards without citric acid resulted in formation of suspended particles, insoluble precipitate, or dried solute scum layer on the glassy surface, causing losses

Table 1. Migration of some heavy metal compounds in different solvent systems [a]

Sample	Concn, μg/20 μL	R_f values (distd water stds not concd)			Concd distd or fresh water stds,
		0.1% NaCl	0.5% NaCl	1% NaCl	0.1% NaCl
Paper Chromatography					
$HgCl_2$	2	0.86 ± 0.05	0.88 ± 0.04	0.93 ± 0.05	0.85 ± 0.039
$CuSO_4$	2	0.5 ± 0.025	0.78 ± 0.04	0.85 ± 0.042	0.95 ± 0.042
$AgNO_3$	2	0.1 ± 0.005	0.03 ± 0.0012	—	—
$CdSO_4$	5	0.93 ± 0.05	0.95 ± 0.05	0.97 ±0.05	0.95 ±0.05
Micro TLC					
$HgCl_2$	2	0.83 ± 0.04	0.92 ± 0.05	0.95 ± 0.05	0.8 ± 0.042
$CuSO_4$	2	0.17 ± 0.009	0.18 ± 0.01	0.25 ± 0.013	0.09 ± 0.005
$AgNO_3$	2	—	—	—	—
$CdSO_4$	5	0.5 ± 0.028	0.67 ± 0.034	0.92 ± 0.05	0.6 ± 0.029

[a] Mean ± SD for 6 observations.

of compounds. Adding citric acid alleviated these problems.

$HgCl_2$ could be concentrated only through evaporation. This compound could not be precipitated by pH 8.2 sodium phosphate buffer but requires citric acid addition and neutralization with an equal volume of pH 7.2 buffer. $AgNO_3$ could be precipitated by phosphate buffer but the residue could not be redissolved by citric acid. Hence $AgNO_3$ was concentrated by evaporation to the required volume in citric acid and neutralized with pH 7.2 buffer.

Citric acid is a mild bio-acid and in the ratio of 1:9 (2% citric acid:pH 7.2 phosphate buffer) does not affect the enzyme, whereas higher concentrations are inhibitory. Therefore, $CdSO_4$ residue, dissolved in citric acid and diluted with distilled water, is subjected to buffer treatment by repeated spraying on paper or TLC plate. Preparation of enzyme in pH 7.2 buffer also helps neutralize acidity of citric acid. The limitation in the above concentration method is that it cannot be used for analysis by atomic absorption spectroscopy because the substances might cause non-atomic absorption, light scattering, or reduction of nebulizer efficiency (8).

The heavy metal compounds can be separated by using 0.1% NaCl as a solvent system (Table 1). The varying mobilities of the heavy metals may be due to co-migration with NaCl. Distilled water or 0.5 and 1% NaCl solvent did not separate the heavy metals.

After enzymatic color reaction, heavy metals appear as white inhibition zones (1, 2). These zones represent the inhibition caused by heavy metals on dehydrogenase (1, 2) and the subsequent non-reduction of tetrazolium salt (INT) to farmazon (1, 2). The present method is simpler than the method reported earlier (1) because the laborious use of a moist atmospheric chamber is avoided. The paper strips or TLC plates are placed directly in the hot air oven. The moisture present on the paper and plates is enough for enzymatic color reaction. Up to 3% NaCl does not affect the succinate dehydrogenase system, causing neither inhibition nor color reaction. Non-interference by NaCl has a great advantage. For example, when water samples with low heavy metal content are evaporated, the concentrated salt does not interfere.

The R_f values of $CuSO_4$ analyzed from distilled water standards are different from fresh water standards. The method of standard additions (8) may be followed by adding a known amount of $CuSO_4$ to the fresh water samples and then matching R_f values. For better separation and detection of these metal compounds, either micro TLC or paper chromatography or both together have to be employed (Table 2). The earlier method reported (1) is suitable for identifying a single compound whose case history is known either for fresh water analysis or for routine laboratory detection. The present method with 0.1% NaCl as solvent system is suitable for mul-

Table 2. Recommended chromatographic methods for separation and detection of some heavy metals, 0.1% NaCl solvent system

Compound	Chromatographic method recommended
$CuSO_4$	micro TLC
$CdSO_4$	paper chromatography
$HgCl_2$	paper or micro TLC
$AgNO_3$	paper or micro TLC
$CuSO_4$–$CdSO_4$ mixture	TLC
$AgNO_3$–$HgCl_2$ mixture	paper or micro TLC
$CuSO_4$–$HgCl_2$ mixture	paper chromatography
$CdSO_4$–$HgCl_2$ mixture	paper chromatography

timetal identification and separation at 40 ppm (cadmium is detectable at 500 ppm). Screening can be made on a detector strip (2) or on a micro TLC plate (1) at nanogram concentrations. Preferred methods are listed in Table 2.

The method is suitable for identification only of ionized free metal compounds which fall within the detection limits, not for metal complexes or colloidal forms. The metal compounds stannous chloride, ammonium arsenite, aluminum chloride, chromium sulfate, magnesium chloride, selenium chloride, cobalt nitrate, barium chloride, lead nitrate, zinc chloride, strontium chloride, and ammonium molybdate have no effect. Barium and zinc chloride cause inhibition at very high amounts (200 μg) but interference is overcome through simultaneous matching with known standards.

Acknowledgments

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PESTICIDE RESIDUES

Determination of 2,4-D Butoxyethanol Ether Ester and Its Degradation Products 2,4-Dichlorophenoxyacetic Acid and 2,4-Dichlorophenol in Sediment

GORDON Y. P. KAN, FRED T. S. MAH, NORMAN L. WADE, and MAX L. BOTHWELL[1]
Environment Canada, Inland Waters Directorate, Water Quality Branch, 1305 Welch St, North Vancouver, British Columbia, Canada V7P 1B3

2,4-D butoxyethanol ether ester (2,4-D BEE) was isolated from sediment by extraction with acetone-hexane at neutral conditions; from the same sediment sample, 2,4-dichlorophenoxyacetic acid (2,4-D) was isolated with methanol after the sediment had been acidified to < pH 2. 2,4-Dichlorophenol in the sediment was extracted with isooctane by steam distillation. Mean recoveries from 4 spiked samples ranged from 99 to 108%, 94 to 102%, and 74 to 76% for 2,4-D BEE, 2,4-D, and 2,4-dichlorophenol, respectively. These procedures were developed for routine analyses of lake sediments in the Okanagan region of British Columbia.

Chlorophenoxy herbicides have been used across Canada for over 25 years to control terrestrial broad-leaved weeds (1). Recently, the appearance of the nuisance weed, Eurasian milfoil (*Myriophyllum spicatum*), in the Okanagan lakes of British Columbia prompted the use of 2,4-D butoxyethanol ether ester (2,4-D BEE). To monitor the possible persistence of herbicide residues in the environment, it became necessary to develop sensitive and reliable methods for determining 2,4-D BEE and its degradation products, 2,4-D and 2,4-dichlorophenol. Although analytical methods for a wide range of chlorophenoxy herbicides have been reported for soil, water, and tissue samples (2–11), no single publication has been found that provides procedures for the coordinated determination of 2,4-D BEE and its degradation products in wet sediment. Most published methods simply report on the hydrolysis of the 2,4-D esters to the corresponding acids or salts, or extraction of samples with various solvents, or chemical derivatization and gas chromatographic identification and quantitation (12–14).

Because 2,4-dichlorophenol is highly volatile, solvent extraction becomes impractical due to losses by co-distillation when raw extracts are subjected to concentration on a rotary evaporator both before and after derivatization (G. Y. P. Kan & R. So (1980) unpublished results). Continuous liquid-liquid extraction is also not accurate because of solute loss during concentration (15). Furthermore, exhaustive solvent extraction procedures co-extract lipids, waxes, pigments, and other high molecular weight organics, so extensive chromatographic cleanup of the raw extracts is often necessary before injection into a gas chromatograph for trace analysis. On the other hand, steam distillation, a classical method of separating chemicals on the basis of differences in vapor pressures over water (16), may provide a simple and convenient procedure for the extraction of 2,4-dichlorophenol from wet sediment. For this reason, we have adapted a modified Nielsen-Kryger steam distillation apparatus (17) for the extraction of 2,4-dichlorophenol from sediment.

Because of rapid hydrolysis, it is not possible to isolate 2,4-D BEE by steam distillation. However, 2,4-D BEE can be satisfactorily extracted from sediment at neutral conditions by using a mixture of acetone and hexane. When the extraction of 2,4-D BEE is completed, the sediment can be acidified and 2,4-D can be extracted quantitatively with methanol.

Experimental

Apparatus

(a) *Gas chromatographs.*—(*1*) MT-220 equipped with linearized ^{63}Ni electron capture detector; 1.8 m × 4 mm id glass column packed with 3% OV-101 on 80–100 mesh Chromosorb W (Chromatographic Specialties Ltd, Brockville, Ontario).

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[1] National Water Research Institute, Pacific and Yukon Branch, Inland Waters Directorate, Environment Canada, 4160 Marine Dr, West Vancouver, British Columbia, Canada V7V 1N6.

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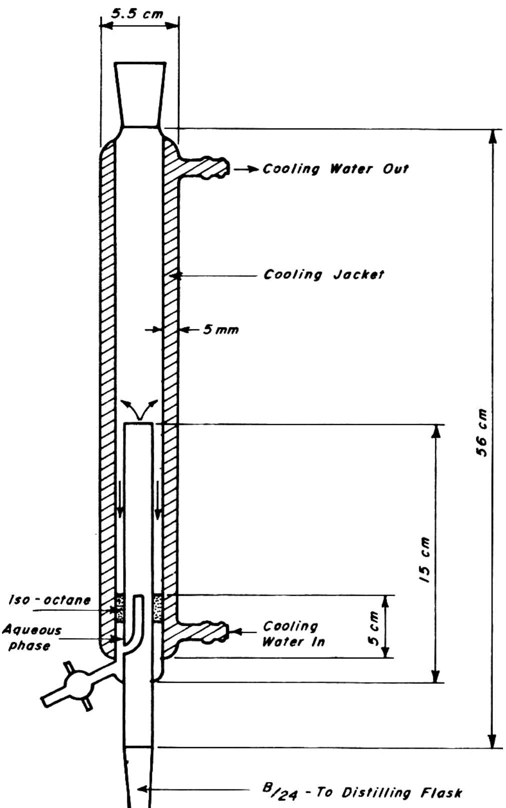

Figure 1. Steam distillation apparatus; modified design of Veith and Kiwus (16).

Operating conditions: injector 190°C, column temperature 200°C, detector temperature 350°C, carrier gas 5% methane in argon flowing at 65 mL/min. (2) Hewlett-Packard Model 5750 equipped with ^{63}Ni electron capture detector; 1.8 m × 4 mm id glass column packed with 1% SP-1240 on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA). Operating conditions: injector 190°C, column temperature 100°C, detector 350°C, 5% methane in argon carrier gas flowing at 55 mL/min.

(**b**) *Steam distillation column.*—Modified design of Veith and Kiwus (16); see Figure 1.

(**c**) *Shaker.*—Eberbach rotating, variable speed shaker (Canlab, Division of McGaw Supply Ltd).

(**d**) *Test tube mixer.*—Vortex-Genie, Fisher Scientific Co. Ltd.

(**e**) *Rotary evaporator.*—Rotavapor-R evaporator (Buchi, Fisher Scientific Co. Ltd).

Reagents

(**a**) *Diazomethane reagent.*—Prepared from Diazald, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Co.), in ethyl ether according to manufacturer's instruction and preparation kit.

(**b**) *Solvents.*—All solvents used were pesticide grade (Burdick & Jackson Laboratories, Inc.).

(**c**) *Chemical standards.*—All standard compounds were obtained from Environmental Protection Agency, Research Triangle Park, NC. Stock standards were prepared with acetone; working standards were prepared with isooctane.

(**d**) *Florisil.*—60–100 mesh (Fisher Scientific Co.), heat-treated 4 days at 650°C and deactivated with 0.5% water (18).

(**e**) *Glass fiber filter.*—Whatman GF/C, heat-treated overnight at 325°C.

Sample Collection

The sediment was collected in Kalamalka Lake, British Columbia, in early summer 1979 and stored at 4°C in glass jars. It had an average moisture content of about 26%, organic carbon content of about 1% (dry wt), and pH of 7.8 for its associated water. For the recovery study, 25 g wet sediment was placed in a 250 mL volumetric flask with the aid of a spatula and powder funnel. Ten mL of an intermediate standard (ppm range) in acetone was added to the sediment. Distilled water, 5–8 mL, was used to wash down any sediment that might stick on the neck of the flask. The sediment was then swirled so that the spiking chemicals were uniformly distributed. Organic solvents were added for extraction. For steam distillation, the sediment was fortified with 2,4-dichlorophenol in the distillation flask in a similar manner. All extracts were diluted to 10 mL for GLC analysis.

Extraction

(**a**) *Solvent extraction of 2,4-D BEE.*—Place 25 g wet sediment in 250 mL volumetric flask; add 40 mL acetone and 5 mL distilled water. Mix on shaker 20 min. Add 80 mL hexane and resume shaking for another 20 min. Vacuum-filter mixture through heat-treated glass fiber filter. Return sediment from filter to volumetric flask and re-extract with mixture of 20 mL acetone and 40 mL hexane for another 20 min. Filter sediment and repeat this re-extraction process once more. Return sediment on filter to volumetric flask for extraction of 2,4-D. Transfer combined acetone–hexane filtrates to 250 mL separatory funnel. Let phases separate and return aqueous phase to filtering flask for extraction of 2,4-D [see *Extraction* (**b**)]. Drain acetone–hexane into 250

mL round-bottom flask; then add 5 mL isooctane. Strip acetone-hexane from extract on rotary evaporator and reduce volume to ca 0.5 mL. Transfer concentrated extract to 15 mL graduated centrifuge tube with the aid of isooctane and dilute to 10 mL for GLC analysis on 3% OV-101 column.

(b) *Solvent extraction of 2,4-D.*—Add 5 mL distilled water, 8 mL sulfuric acid (50%), and 50 mL methanol to sediment derived from previous extraction (a). Check pH of slurry which should be below 2. Shake mixture 20 min on shaker. Filter mixture and pour filtrate into flask that contains aqueous phase saved from previous extraction (a). Re-extract sediment with 50 mL methanol for another 20 min. Transfer combined filtrate to 250 mL round-bottom flask and flash-evaporate until only aqueous fraction remains. Then add 50 mL methylene chloride and shake briefly. Empty mixture into 250 mL separatory funnel and shake vigorously ca 1 min. When phases separate, drain methylene chloride into Erlenmeyer flask. Re-extract aqueous phase with another 25 mL methylene chloride. Dry combined organic extract over anhydrous sodium sulfate. Carefully decant extract into 150 mL round-bottom flask; rinse sodium sulfate with 5 mL isooctane. Concentrate extract and isooctane wash to ca 0.5 mL. Cool flask under cold water tap or in ice bath. Add diazomethane dropwise until yellow color persists; generally 0.5 mL will suffice. Do *not* transfer extract to smaller container such as 15 mL centrifuge tube for derivatization. Perform derivatization in same round-bottom flask to prevent any unpredictable loss of 2,4-D due to adsorption on flask (G. Y. P. Kan et al., manuscript in preparation). After addition of diazomethane, let mixture stand 5–10 min at room temperature. Remove excess diazomethane by blowing with nitrogen. Transfer product with isooctane to 15 mL graduated centrifuge tube and dilute to 10 mL for GLC analysis on a 3% OV-101 column.

(c) *Extraction of 2,4-dichlorophenol by steam distillation.*—Introduce 25 g wet sediment into 500 mL round-bottom flask; add 100 mL distilled water and 15 mL isooctane. Attach distillation column to flask (Figure 1) and gently reflux mixture 2 h. Cool flask, drain aqueous phase trapped in distillation column, and collect isooctane distillate in 15 mL graduated centrifuge tube. Rinse distillation column with some isooctane. Pool rinses and dilute to 15 mL; then vortex-mix with 2–5 drops of metallic mercury to remove sulfurous materials before GLC analysis on 1% SP-1240 column.

Cleanup of 2,4-D and 2,4-D BEE

Raw extracts of sediment samples usually contain numerous co-extractives and artifacts which have GLC responses similar to the herbicides in question. These interferences, however, may be conveniently removed by means of a Florisil column.

Florisil cleanup column.—Place plug of glass wool into 23 cm disposable pipet. Pack pipet with ca 3 g Florisil. Place 2–4 mm anhydrous sodium sulfate on top of Florisil. Wet column with petroleum ether (30–60°C). Place 15 mL graduated centrifuge tube under pipet as receiver. Transfer derivatized raw extract quantitatively onto column with some petroleum ether. Elute column with 15 mL petroleum ether; discard this eluate. Next elute column with 10 mL ethyl ether–petroleum ether (1 + 1) into 15 mL graduated centrifuge tube. Concentrate eluate with dry nitrogen to near dryness and dilute to 10 mL with isooctane for GLC analysis. Recoveries of 2,4-D methyl ester and 2,4-D BEE range from 95 to 100%.

Results and Discussion

Mean recoveries from wet sediment samples spiked with low and high doses of 2,4-D BEE, 2,4-D, and 2,4-dichlorophenol ranged from 99 to 108%, 94 to 102%, and 74 to 76%, respectively (Table 1). While 2,4-D BEE could be isolated satisfactorily by solvent extraction using a mixture of acetone and hexane, 2,4-D was not extractable from sediment unless the medium was acidified to pH < 2 with sulfuric acid. Methanol was used in the extraction of 2,4-D because it was a polar solvent and would readily wet the surface of the sediment particles in the presence of aqueous acid. For derivatization, it was necessary to back-extract the 2,4-D from the aqueous methanol fraction with methylene chloride. It is essential to remove the methanol from the aqueous phase before addition of methylene chloride because methanol is soluble in both methylene chloride and water and thus it would interfere with the partitioning of 2,4-D into methylene chloride.

Methylene chloride in the extract, in turn, had to be displaced with isooctane before derivatization because it would saturate the electron capture detector of the gas chromatograph. Derivatization with diazomethane was performed in the same round-bottom flask that was used to concentrate the isooctane extract to prevent losses of 2,4-D due to adsorption on the glass (G. Y. P. Kan et al., manuscript in preparation).

Table 1. Recovery (%) of 2,4-dichlorophenol, 2,4-D BEE, and 2,4-D from 25 g wet sediment

Sample	2,4-Dichlorophenol added, µg		2,4-D added, µg		2,4-D BEE added, µg	
	0.4	2.5	0.2	2.0	0.4	4.0
1	72	75	83	104	111	91
2	72	78	100	103	111	100
3	78	75	100	100	104	108
4	75	75	94	102	106	97
Mean	74.3	75.8	94.3	102	108	99.0
SD	2.87	1.50	8.01	1.29	3.56	7.07
CV, %	3.87	1.98	8.50	1.25	3.29	7.14

From recovery data, it appeared that steam distillation offered a simple procedure for the quantitative determination of 2,4-dichlorophenol in sediment. Because only a small amount of solvent was required during steam distillation, this procedure eliminated any uncontrollable loss of volatile 2,4-dichlorophenol on the rotary evaporator. During steam distillation, a great deal of sulfurous material was co-distilled, which interfered with 2,4-dichlorophenol analysis on the 1% SP-1240 column (19). However, these interferences could be conveniently removed by vortexing the distillate with a few drops of metallic mercury. One advantage of using 1% SP-1240 over 3% OV-101 is that no derivatization of the 2,4-dichlorophenol is necessary. Distillation for 1 h gave only 45–57% recovery, while distillation over a period of 2 h gave 74–75% recovery. Further distillation of the sediment for 1.5 h did not produce additional yield.

To show that 2,4-D BEE and 2,4-D did not contribute to the recovery of 2,4- dichlorophenol during steam distillation, a duplicate sediment sample (25 g) was spiked with 2,4-D BEE (4 µg) and 2,4-D (2 µg) and steam distilled 2 h. However, no trace of 2,4-dichlorophenol was observed from the steam distillate.

Our attempts to isolate 2,4-D BEE by steam distillation failed because this compound rapidly hydrolyzed to 2,4-D once the temperature of the sediment was increased. In fact, the hydrolysis of 2,4-D BEE to 2,4-D under basic or acidic conditions has been well documented (5). Attempts to extract this compound by using methylene chloride failed because of formation of emulsion which presented great difficulties in the subsequent filtration process.

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Determination of Norflurazon Residues in Mixed Crop Matrices

VICTOR W. WINKLER, JAYANTI R. PATEL, MARY JANUSZANIS, and MARJORIE COLARUSSO
Sandoz, Inc., Crop Protection, East Hanover, NJ 07936

A method for determining norflurazon residues in mixed crop matrices is described. The method includes methanol Soxhlet extraction, hexane wash, addition of 10% saline, methylene chloride extraction, concentration, preparative thin layer chromatography of 10% sample aliquot, and resuspension of sample zone for gas-liquid chromatographic analysis. The lower level of electron capture detection is 0.01 ppm. Recoveries for 0.08 ppm spiked samples of cottonseed; soybean seed and foliage; peanut seed plus hulls and foliage; orange and tangerine; lemon; apple and peach; and soil are generally greater than 80%.

Norflurazon, 4-chloro-5-(methylamine)-2-(α,α,α-trifluoro-*m*-tolyl)-3-(2*H*)-pyridazinone, is a pre-emergence herbicide for cotton, cranberries, fruits, and nuts. Crop residues consist of norflurazon and its desmethyl metabolite. A combination of several methods developed for residue analysis of several individual crop substrates is described by Brady et al. (1). However, proposed label expansion for use on other crops such as soybeans, peanuts, and citrus requires the development of a norflurazon residue method applicable for all substrate matrices.

METHOD

Reagents

Solvents must be pesticide grade or equivalent. Suitable products are available from Burdick & Jackson Laboratories, Inc., and other manufacturers. See *Official Methods of Analysis* (2) for solvent purity tests.

(a) *Desmethyl norflurazon standard.*—99+% pure (Sandoz, Inc.)

(b) *Norflurazon standard.*—99+% pure (Sandoz, Inc.)

(c) *Phosphoric acid solution.*—0.01% in methanol.

(d) *Sodium chloride solution.*—10% in deionized water.

(e) *TLC plates.*—20 × 20 cm, silica gel GF 254, 250 µm (Analtech).

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Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5700, or equivalent, with ^{63}Ni electron capture detector and 1 m × 2 mm id glass column packed with 0.15% Poly A 103 plus 3% SE-30 mixed phase on Chromosorb W(HP) (Applied Science Laboratories). Operating conditions: temperatures (°C)—column 200, injection port 250, detection block 350; carrier gas 5% argon-methane; flow rate 30 mL/min.

(b) *Soxhlet extractor.*—Equipped with hot plate and 35 × 94 mm extraction thimbles or equivalent.

Preparation of Standards

(a) *Norflurazon stock solution.*—1 mg/mL. Dissolve 100 mg norflurazon in methanol and dilute to 100 mL with same solvent.

(b) *Desmethyl norflurazon stock solution.*—1 mg/mL. Dissolve 100 mg desmethyl norflurazon in methanol and dilute to 100 mL with same solvent.

(c) *Norflurazon plus desmethyl norflurazon working solution A.*—10 µg/mL admixture. Dilute 1 mL each of norflurazon and desmethyl norflurazon, 1 mg/mL stock solution, to 100 mL with methanol.

(d) *Norflurazon plus desmethyl norflurazon working solution B.*—1 µg/mL admixture. Dilute 10 mL working solution A to 100 mL with methanol.

(e) *Norflurazon plus desmethyl norflurazon standard curve solutions.*—0.1, 0.08, 0.06, 0.04, and 0.02 µg/mL admixture in 0.01% methanolic H_3PO_4. Dilute the 1 µg/mL working solution B with 0.01% methanolic H_3PO_4 as shown below:

Concn, µg/mL	*Working Soln B*	*0.01% H_3PO_4 in Methanol*
0.10	10	90
0.08	8	92
0.06	6	94
0.04	4	96
0.02	2	98

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Extraction of Sample

Weigh 25 g chopped sample into extraction thimble. For 0.08 ppm standard addition recovery (spiked sample), add 2 mL 1.0 μg/mL admixture working solution B to sample in extraction thimble and let solution dry. Soxhlet extract overnight with 200 mL methanol and glass beads. Filter methanolic extract through glass wool to remove solid particles. Wash flask with 25 mL methanol and combine filtrates in 500 mL separatory funnel. Extract lipid material from methanol solution 2 times with 200 mL hexane each. For soil samples, add 25 mL deionized water to obtain efficient phase separation of hexane and methanol. Add 150 mL 10% aqueous NaCl to lower methanolic phase and re-extract with 100 mL hexane. Discard hexane phase. Extract (non-vigorously) norflurazon residues from aqueous methanolic solution 3 times with 100 mL methylene chloride each time. Pass each methylene chloride extract through funnel plugged with glass wool and containing anhydrous Na_2SO_4 into 500 mL evaporating flask and dry by rotary evaporation at 50°C. Resuspend residue in methanol–methylene chloride (1 + 1) and dilute to 5 mL in graduated centrifuge tube. Mix resuspended residue and, if necessary, centrifuge at moderate speed to obtain a clear supernate for preparative thin layer chromatographic (TLC) cleanup.

Preparative TLC Cleanup

Mark two 2 cm runways on each side of 20 × 20 cm silica gel GF 254 TLC plate. Apply 0.5 mL aliquot of resuspended sample as 16 cm zone between runways, and spot ca 1 μg mixed norflurazon plus desmethyl norflurazon standards on each runway for reference. Develop plate to 12 cm in paper-lined equilibrated chamber with methylene chloride–ethyl acetate (1 + 1). Remove plate, air-dry, and locate mixed norflurazon and desmethyl norflurazon reference standard (co-chromatographed R_f ca 0.5) under 254 nm light. Remove sample zone with razor blade and resuspend in 2.5 mL 0.01% H_3PO_4 in methanol. Remove silica gel on plate above sample zone before removing sample zone. Vigorously mix resuspended sample and centrifuge to obtain clear supernate for gas-liquid chromatographic (GLC) analysis.

Chromatography and Calculations

Adjust GLC conditions to give ca 50% pen response for 2 μL injection of 0.1 μg/mL admixture standard B with R_t ca 3 and 5 min, respectively.

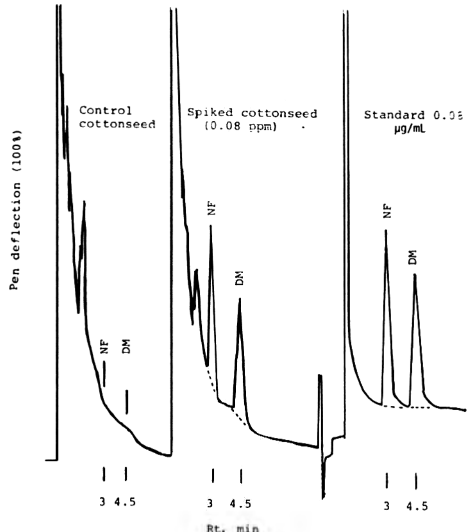

Figure 1. Representative chromatograms of norflurazon (NF) and its desmethyl metabolite (DM) residues.

Inject 2 μL standard curve solutions after constant conditions are obtained and prepare standard curve of μg norflurazon and desmethyl norflurazon/mL vs respective peak height response. After linearity has been established, inject 2 μL sample solution and determine respective norflurazon and desmethyl norflurazon peak height response. If necessary, dilute sample solutions accordingly for peak height response to be in 0.1 to 0.02 μg/mL standard curve range.

Determine μg norflurazon and desmethyl norflurazon/mL in sample either (*1*) from standard curve, and convert this value directly to ppm (sample equivalent concentration is 1 μg/mL); or (*2*) use following proportionality if standard curve is linear and passes through origin:

Norflurazon or desmethyl norflurazon, ppm $= (P/P') \times (\mu L \text{ std inj.}/\mu L \text{ sample inj.}) \times [(\mu g/mL \text{ std})/(g \text{ equiv. sample}/mL)]$

where P and P' = peak heights of sample and standard, respectively. Report results as ppm total norflurazon plus desmethyl norflurazon.

Results and Discussion

Representative chromatograms of norflurazon residue analysis in a non-treated cottonseed sample, the corresponding 0.08 ppm spiked sample, and 0.08 μg/mL mixed standard are shown in Figure 1. There is excellent norflurazon and desmethyl norflurazon separation and

Table 1. Recoveries of 0.08 ppm norflurazon (NF) and desmethyl norflurazon (DM) standard addition of various non-treated crop substrates

Substrate	*n*	Mean % recovery ± CV		
		NF	DM	NF + DM, Av.
Cottonseed	6	99 ± 26	100 ± 34	99 ± 28
Soybean seed	9	93 ± 13	99 ± 18	95 ± 17
Soybean foliage	8	94 ± 23	112 ± 30	103 ± 16
Peanut seed + hull	3	94 ± 29	108 ± 19	101 ± 24
Peanut foliage	5	82 ± 11	77 ± 17	79 ± 12
Orange and tangerine	6	90 ± 17	97 ± 13	94 ± 15
Lemon	8	80 ± 12	90 ± 12	85 ± 12
Apple and peach	4	101 ± 35	88 ± 16	94 ± 28
Soil	9	104 ± 25	94 ± 27	99 ± 25

near baseline resolution with cottonseed which is typically more difficult to analyze because of its high oil content. The injection solutions contain 0.01% phosphoric acid to preserve the integrity of the GLC column which is stable for about one month of continuous use. The lower limit of detection is about 0.01 ppm.

Recoveries of 0.08 ppm spiked samples assayed as part of various residue studies involving cottonseed; soybean seed and foliage; peanut seed plus hulls and foliage; orange and tangerine; lemon; apple and peaches; and soil are shown in Table 1. Recoveries obtained with these crop substrates are typically greater than 80%.

A major advantage of this method over those previously reported (1) is the applicability for mixed crop matrices and the simplicity of the extraction and preparative TLC over conventional column chromatography. The total turnover time per assay is 24 h including the overnight Soxhlet extraction; an operator can assay 6 samples simultaneously with no difficulty.

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Thin Layer Chromatographic Determination of Methyl Parathion as Paraoxon by Cholinesterase Inhibition

SANA UDAYA BHASKAR and NANGUNERI V. NANDA KUMAR
Sri Venkateswara University, Department of Zoology, Tirupati-517 502, India

A simple, sensitive, and rapid method is described for the quantitative estimation of nanogram amounts of methyl parathion (*O,O*-dimethyl *O-p*-nitrophenyl phosphorothioate) as methyl paraoxon (*O,O*-dimethyl *O-p*-nitrophenyl phosphate) on thin layer chromatograms. Methyl paraoxon is detected by pig liver acetone powder cholinesterase inhibition, using *p*-nitrobenzenediazoniumfluoroborate as the chromogenic reagent. Commercial pig liver acetone powder is more advantageous than raw liver sources because it is readily available and can be preserved indefinitely. About 0.1 ng methyl parathion can be detected, and amounts from 5 to 50 ng can be quantitatively estimated.

Thin layer chromatographic (TLC)-enzymatic methods using cholinesterase (ChE) inhibition techniques for detection (1, 2) and determination (3, 4) specify live animal tissue sources for enzyme. Obtaining and preparing the enzyme reagent is cumbersome and time consuming. Recently, sensitive and simple colorimetric methods using pig liver acetone powder as the enzyme source have been reported (5, 6) for estimating methyl parathion and dimethoate. We report on advantages of pig liver acetone powder compared with other sources, especially raw liver, the detection limits, and the suitability for detecting methyl parathion as methyl paraoxon on TLC plates. Paddy grain samples were fortified with methyl parathion and analyzed by the TLC-enzymatic method described.

METHOD

Apparatus and Reagents

Use analytical grade reagents.

(a) *Gas-liquid chromatograph.*—Varian Aerograph series 1400 equipped with 6 ft × 1/8 in. id Pyrex glass column packed with 5% OV-17 on 60-80 mesh Chromosorb W. Nitrogen carrier gas (24 mL/min). Alkali flame ionization detector with hydrogen flow 45 mL/min and air flow 260 mL/min. Temperatures (°C)—injector 150, column 190, and detector 200. Range 32 × 10^{-12}.

(b) *Methyl parathion.*—Prepare different concentrations of 99% pure methyl parathion (Ciba-Geigy Ltd, Basel, Switzerland) in acetone.

(c) *1-Naphthyl acetate substrate solution.*—Dissolve 20 mg 1-naphthyl acetate (Sigma Chemical Co., St. Louis, MO 63178) in 4 mL acetone.

(d) *p - Nitrobenzenediazoniumfluoroborate.*—Reidel, Budapest, Hungary. Prepare 0.4% in acetone.

(e) *Silica gel G.*—BDH Laboratories, Bombay, India.

Preparation of Enzyme from Liver Acetone Powders

Prepare separate 1% enzyme suspensions of liver acetone powders of pig, beef, and horse (Sigma Chemical Co.) in distilled water (0-5°C) with the help of electrical homogenizer. Filter through 4 layers of cheesecloth.

Preparation of Enzyme from Sheep and Rat Livers

Prepare separate 10% homogenates of fresh sheep and albino Wistar rat livers in distilled water (0-5°C). Filter through 4 layers of cheesecloth and preserve suspension in refrigerator at 5°C before use.

Thin Layer Chromatography

Coat 20 × 10.5 cm glass plates with 450 μm layer of silica gel G in water slurry. Air dry plates and activate 1 h in 110°C oven. Store plates in desiccator before use.

Clean up methyl parathion residues in fortified and field samples of paddy grain by procedure of Mendoza and Shields (7). Concentrate aliquots to obtain equivalent amounts of residues in each extract. Apply different concentrations of methyl parathion standards (5-50 ng) and sample extracts with micropipet, and oxidize methyl parathion to methyl paraoxon as reported previously (3).

Develop plate 15 cm in acetone-hexane (1 + 4). Air dry plate 5 min. Uniformly spray liver acetone powder suspensions or raw liver suspensions over plate as fine mist, thoroughly wetting gel. Keep plate 20 min in moist atmosphere at 37°C. Spray plate with 1-naphthyl acetate substrate solution. Replace plate 2 min in moist atmosphere at 37°C. Uniformly spray

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Table 1. Relationship of enzyme inhibition with methyl parathion concentration as determined by area measurement and area weight methods

Concn, ng/mL	Area measurement method: Inhibition zone, sq. mm	Area measurement method: ChE inhibition, %[a]	Area weight method: Inhibition zone, mg	Area weight method: ChE inhibition, %[a]
5	12.30	5.80 ± 0.92	2.07	3.80 ± 0.61
10	21.00	9.31 ± 1.31	5.10	9.61 ± 0.35
20	43.00	19.11 ± 2.05	10.05	19.23 ± 0.91
30	74.60	32.86 ± 1.86	17.50	32.69 ± 1.85
40	98.50	43.55 ± 2.51	21.00	40.38 ± 1.60
50	128.20	57.32 ± 3.90	27.30	52.49 ± 2.39

[a] Standard deviation for 6 observations.

plate with 0.4% *p*-nitrobenzenediazoniumfluoroborate in acetone and let stand at room temperature. Methyl parathion inhibition of enzyme appears as clear white spot in orange background. Lower limit of detection varies with enzyme source.

Quantitative Estimation of Methyl Parathion

Estimate methyl parathion as methyl paraoxon by area measurement method and area weight method of Nanda Kumar et al. (3).

Quantitative Estimation of Methyl Parathion by GLC

Estimate methyl parathion residues by GLC cleanup according to Storherr et al. (8).

Results and Discussion

With raw rat liver suspension and pig liver acetone powder suspension as enzyme sources, the detection limit for methyl paraoxon was 0.1 ng; with beef and horse liver acetone powders and sheep raw liver suspension, the detection limit was 0.5–1.0 ng. Although rat and pig liver acetone powder suspensions show similar detection limits for methyl paraoxon, the latter has the advantages of ready availability, immediate use, and indefinite storage at 0°C. Enzyme from raw rat liver must be isolated and cannot be stored indefinitely. Compared with other techniques for methyl parathion estimation, detection limits with pig liver acetone powder were comparatively low. Hence the compound is quantitated as methyl paraoxon. Plots of amounts of methyl parathion as paraoxon vs ChE inhibition, both by area measurement and area weight (3), showed a straight line relationship (Table 1). From these standard curves, 5–50 ng methyl parathion can be estimated.

Methyl parathion was extracted from fortified and field samples of paddy grain by the cleanup technique of Mendoza and Shields (7) and estimated by the present method (Table 2). These values compared favorably with the values obtained by GLC.

Table 2. Determination of methyl parathion residues in fortified and field samples of paddy grain by TLC–enzymatic method and GLC

Paddy grain, g	Methyl parathion fortified, μg	TLC–enzymatic method: Area measurement method, μg[b]	TLC–enzymatic method: Area weight method, μg[b]	GLC, μg[c]
50[a]	5	4.75 ± 0.65	4.70 ± 0.60	4.90
50[a]	10	8.95 ± 1.25	9.10 ± 1.20	9.80
50[a]	15	13.80 ± 2.05	13.95 ± 1.95	14.48
50[a]	20	19.20 ± 1.95	19.05 ± 1.80	20.50
500[d]	—	3.90 ± 0.91	4.02 ± 1.05	4.20
500[d]	—	10.21 ± 0.23	9.85 ± 1.20	10.52

[a] Samples were sprayed under laboratory conditions and analyzed after 48 h. The concentration spotted on TLC plate was 5 μL/5 mL hexane.
[b] Standard deviation for 6 observations.
[c] Mean of duplicate observations.
[d] Market samples.

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PESTICIDE FORMULATIONS

Gas-Liquid Chromatographic Determination of Pirimicarb in Formulations: Collaborative Study

PETER D. BLAND
ICI Americas Inc., PO Box 208, Goldsboro, NC 27530

Collaborators: J. Bagness; W. Black; L. Brown; M. Gentry; B. Ginther; E. Hayes; P. Holland; S. Jackling; J. Padmore; L. Torma; G. Winstead

A method is described for the determination of pirimicarb (2-(dimethylamino)-5,6-dimethyl-4-pyrimidinyl dimethylcarbamate) in formulated products by gas-liquid chromatography (GLC). Samples are dissolved in a chloroform solution of an internal standard and injected into a gas chromatograph equipped with a flame ionization detector. Quantitative data are obtained by comparing peak areas of the compound and internal standard with those obtained by injecting a standard solution. Eleven collaborators made replicate determinations on 6 samples including a technical product, a granular product, and 4 powder products. The average coefficient of variation was 1.14% for the technical product, 1.82% for the granular product, and 0.73% for the powder products.

Pirimicarb (2-(dimethylamino)-5,6-dimethyl-4-pyrimidinyl dimethylcarbamate) is a fast-acting selective aphicide of moderate mammalian toxicity. It is the active ingredient of the products Pirimor® and Aphox® (registered trademarks of Imperial Chemical Industries Ltd, London, UK). Pirimor and Aphox have proved effective against organophosphorus-resistant aphids. In addition to contact action, Pirimor spray has a fumigant effect and its persistence in treated crops is short (1).

Methods of analysis for pirimicarb involving titrimetry, colorimetry, and gas-liquid chromatography are described in the literature (2, 3). This collaborative study is based on a gas-liquid chromatographic method described by Bagness and Sharples (3).

Pirimicarb – Gas-Liquid Chromatographic Method – Official First Action

Principle

Pirimicarb is detd by gas-liq. chromatgy, using nonadecane as internal std and flame ionization detection. Peak areas are compared with that of std of known purity.

Apparatus

(a) *Gas chromatograph.*—With heated, glass-lined, injection port and flame ionization detector. Conditions given are for Hewlett Packard Model 5710A. Other instruments may require changing operating parameters to obtain good resolution and response. Temps (°)—column 210, injection port 240, detector 250; gas flow rates (mL/min)—N carrier gas 40, H 60, air 240; attenuation 32 × 10; sample size 1.0 μL; retention times (min)—pirimicarb 6.8, internal std 8.9. Adjust parameters to assure complete sepn of peaks, and peak hts ca 60–80% full scale on chart at quoted retention times.

(b) *Column.*—1.8 m (6 ft) × 0.25 in. (od) × 2 mm (id) glass column packed with 10% silicone SE-30 on 100–120 mesh Chromosorb W(HP) (Applied Science Laboratories, Inc.). Silanize with 30 μL Silyl 8 (Pierce Chemical Co., PO Box 117, Rockford, IL 61105) and heat to 300° for 16 h before use.

Reagents

(a) *Nonadecane internal std soln.*—Accurately weigh ca 1 g nonadecane (Aldrich Chemical Co., Cat. No. N2890-6) and dissolve in 100 mL $CHCl_3$. Store in tightly capped bottle to avoid evapn. Check internal std soln for interfering components by injecting 1 μL into chromatograph.

(b) *Pirimicarb std soln.*—Accurately weigh ca 150 mg pirimicarb std of known purity (ICI Americas Inc., PO Box 208, Goldsboro, NC 27530) into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve pirimicarb. Store tightly capped to avoid evapn.

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The recommendation of the Associate Referee was approved by the General Referee and Committee A and adopted by the Association. See *J. Assoc. Off. Anal. Chem.* **65**, March issue (1982) for detailed reports.
This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.

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Table 1. Collaborative results for GLC determination of pirimicarb (% active ingredient)

	Sample 1 technical		Sample 2 powder		Sample 3 powder		Sample 4 powder		Sample 5 powder		Sample 6 granule	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	97.67	96.75	50.00	50.05	49.83	49.83	47.56	47.48	45.66	45.58	48.75	48.50
	96.96	97.45	50.05	49.97	49.90	49.85	47.47	47.51	45.59	45.58	48.77	48.54
Mean	97.32	97.10	50.03	50.01	49.87	49.84	47.52	47.50	45.63	48.58	48.76	48.52
2[a]	99.44	98.56	50.83	49.27	50.56	50.05	48.96	48.94	48.69	46.94	50.89	50.01
	100.72	96.97	50.89	49.53	51.34	49.95	49.29	47.75	47.86	47.80	51.16	50.46
Mean	100.08	97.76	50.86	49.40	50.95	50.00	49.13	48.34	48.28	47.37	51.03	50.24
3[b]	97.88	97.43	50.06	49.18	49.43	49.12	47.00	46.77	45.68	45.36	49.07	49.25
	97.88	96.75	50.25	50.15	49.38	49.59	47.65	47.12	45.11	45.31	49.07	50.16
Mean	97.88	97.09	50.16	49.67	49.41	49.36	47.33	46.95	45.40	45.34	49.07	49.71
4[c]	97.98	97.84	50.34	50.31	50.11	49.91	47.79	47.52	45.67	45.56	48.35	48.03
	97.98	97.87	50.35	50.29	50.12	49.88	47.79	47.50	45.65	45.56	48.35	48.03
Mean	97.98	97.85	50.34	50.30	50.11	49.90	47.79	47.51	45.66	45.56	48.35	48.03
5	96.01	98.51	50.13	49.91	49.74	49.55	47.34	47.98	45.56	46.00	48.72	47.92
	95.93	98.43	50.08	49.87	49.72	49.61	47.41	48.07	45.54	45.99	48.77	47.92
Mean	95.97	98.47	50.11	49.89	49.73	49.58	47.38	48.03	45.55	46.00	48.75	47.92
6[d]	95.03	98.07	50.19	50.19	50.11	49.81	47.45	47.55	45.29	45.57	48.23	48.42
	95.60	98.13	50.32	50.24	50.14	49.87	47.44	47.54	45.30	45.61	48.24	48.42
Mean	95.32	98.10	50.26	50.22	50.13	49.84	47.45	47.55	45.30	45.59	48.24	48.42
7	98.85	98.09	50.57	49.88	49.95	49.80	48.49	47.63	45.89	45.59	47.68	47.91
	97.93	98.36	50.56	49.95	50.56	50.02	48.06	47.41	45.71	45.99	47.89	48.52
Mean	98.39	98.22	50.57	49.91	50.25	49.91	48.27	47.52	45.80	45.79	47.78	48.22
8[e]	98.02	98.69	50.44	50.01	49.97	49.67	47.76	47.23	46.07	45.76	49.96	49.72
	98.01	98.23	49.80	49.78	49.79	49.67	47.56	47.45	45.89	45.85	49.93	49.36
Mean	98.01	98.46	50.12	49.90	49.88	49.67	47.66	47.34	45.98	45.81	49.94	49.54
9[f]	97.93	97.73	49.93	50.28	49.63	49.65	47.48	47.66	45.33	45.53	48.91	47.80
	97.95	97.96	49.91	50.09	49.78	49.63	47.54	47.43	45.52	45.69	48.90	48.04
Mean	97.94	97.84	49.92	50.19	49.71	49.64	47.51	47.54	45.42	45.61	48.91	47.92
10[g]	95.41	95.50	51.30	50.91	50.54	50.42	48.44	48.46	46.47	46.52	50.51	50.44
	94.95	94.48	50.95	50.79	50.79	50.60	48.72	48.23	46.76	46.16	49.96	50.72
Mean	95.18	94.99	51.12	50.85	50.67	50.51	48.58	48.35	46.62	46.34	50.23	50.58
11[h]	97.63	97.59	49.86	49.74	49.96	49.50	47.45	47.23	45.84	45.54	50.00	50.12
	97.70	97.49	50.00	50.26	49.86	49.32	47.44	47.50	45.74	45.31	50.02	50.18
Mean	97.67	97.54	49.93	50.00	49.91	49.41	47.45	47.37	45.79	45.43	50.01	50.15
7[i] Mean	97.11	98.29	49.90	50.33	50.13	49.97	48.36	47.91	45.62	46.08	50.25	49.59

[a] Not included in mean. Detector temp. 225°C; incomplete resolution.
[b] Oven 180°C, injector 236°C, detector 240°C, 4 ft column.
[c] Oven 215°C, injector 230°C.
[d] Injector 220°C, detector 255°C. Sample 1, day 1: half weight used; Sample 6: methanol pretreatment omitted.
[e] Injector and detector 240°C. Peak height data used.
[f] Column 5 ft.
[g] Peak height data used.
[h] Oven 240°C, injector 250°C, detector 275°C.
[i] 3% OV-1 column, untreated. No supporting data supplied; not included in mean.

Determination

(a) *Powder and technical material samples.*—Accurately weigh amt sample contg ca 150 mg pirimicarb into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve pirimicarb. Keep tightly capped to avoid evapn. Allow insoluble inerts to settle before use.

(b) *Granular formulations.*—Grind sample in mortar and pestle or mech. mill. Accurately weigh amt sample contg ca 150 mg pirimicarb

Table 2. Summary of statistics on GLC determination of pirimicarb[a]

Statistic	Sample					
	1	2	3	4	5	6
SD, indiv. meas. made in succession (within-lab. effect)	0.3260	0.2203	0.1459	0.1696	0.1504	0.2134
SD, mean val. from 2 meas. made in succession[b]	0.2305	0.1558	0.1032	0.1199	0.1063	0.1509
SD, mean val. from diff. days (day-to-day effect within lab.)	0.8649	0.2153	0.1724	0.2628	0.1697	0.3719
SD, mean val. by diff. labs repeat. meas. on diff. days (total effect)	1.1099	0.3442	0.3426	0.3929	0.3305	0.8902
Sample mean, % pirimicarb	97.37	50.18	49.87	47.63	45.71	48.95
CV, %	1.140	0.686	0.687	0.825	0.723	1.819

[a] Based on 10 labs injecting 2 aliquots in succession on 2 different days.
[b] SD of mean = SD of individual/$\sqrt{N}$.

into vial. Add 5.0 mL MeOH and mix to release pirimicarb. Add 10.0 mL internal std soln, cap, and shake to dissolve pirimicarb. Store tightly stoppered to avoid evapn. Allow insoluble inerts to settle before use.

Inject 2 or more aliquots of std soln to set integration parameters and stabilize instrument. Monitor response factor until results agree within 2%. Inject 4 aliquots of std soln and 2 aliquots of sample soln in succession. Calc. response factor, R, for each:

R = area pirimicarb peak/area internal std peak

Pirimicarb, % = $(R/R') \times (W'/W) \times P$

where R and R' = av. response factor for sample and std solns; W and W' = mg sample and std; and P = purity (%) of std.

Results and Discussion

Eleven collaborators analyzed 6 samples. These included one technical sample, one granular sample, and 4 wettable powder samples. Sample 4 was an exact dilution of Sample 3 with inert filler, 96:4. Sample 5 was an exact dilution of Sample 4 with inert filler, 96:4. Collaborators were supplied with column packing, Silyl 8, nonadecane, and pirimicarb standard. They were requested to pack a column, silanize and condition it, and analyze each of 6 samples, using duplicate injections. They were requested to repeat the whole operation on a subsequent day and submit all data to the Associate Referee.

All laboratories except 2 were equipped with electronic integration equipment and submitted area data. Two laboratories submitted peak-height data. The data from all laboratories was recalculated from raw data to check for arithmetical errors and to standardize on rounding. All laboratories followed the instructions closely. Some laboratories used different column lengths and different temperatures to suit their particular instruments. One laboratory omitted the methanol pretreatment of the granular product. One laboratory supplied additional data on an alternative column packing 3% OV-1. Results were not included in the standard deviation calculations but showed good agreement, suggesting that this packing can be used as an alternative.

Results are tabulated in Table 1. The data were analyzed statistically, and 98% confidence limits were calculated for each sample. Collaborator 2 had several outlying results, mostly on day 1, suggesting a problem on that day. Standard deviations were recalculated omitting data from Collaborator 2 and are listed in Table 2. Standard deviations were calculated from pairs to determine within-laboratory effect and day-to-day effect by using the method in reference 4 (p. 18, formula 3).

Recommendations

It is recommended that the gas-liquid chromatography method for the determination of pirimicarb in technical and formulated material be adopted official first action. The lowest concentration tested was 50% ai. Only solid formulations were considered. Liquid formulations of pirimicarb in agriculture are rarely used and are uneconomical because only low strength products are feasible.

Test samples of formulations without internal standard added should be checked by users for interfering components.

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FERTILIZERS

Microwave Oven Drying in the Gravimetric Phosphorus Method

JAMES R. MELTON and WILLIAM L. HOOVER
Texas Agricultural Experiment Station, College Station, TX 77843

The gravimetric quinolinium molybdophosphate method for determining P_2O_5 in fertilizers, AOAC 2.026-2.028, has been modified to permit the precipitate to be dried in a microwave oven rather than a conventional drying oven. After the yellow quinolinium molybdophosphate precipitate is formed, cleaned up, and washed, it is placed in a microwave oven at 600 watts until dry. The quantity of precipitate remaining after microwave drying was equal to that obtained by drying according to AOAC 2.028 for several fertilizer samples. A distinct advantage of the described method is that the yellow precipitate will not char with reasonably excessive drying. Also, much less energy is expended with the microwave oven than with the drying oven.

Since its introduction in 1951, the volumetric method of Wilson (1, 2), in which phosphorus is determined after isolation as quinolinium molybdophosphate, has undergone many modifications. Wilson's precipitating solutions included a citric–molybdic acid reagent and quinoline solution. Fernlund and Zechner (3) eliminated the use of citric acid and prepared one solution which combined sodium molybdate, quinoline, and HCl.

The volumetric quinoline molybdate method was adapted to a gravimetric technique by Perrin (4, 5) for determining total and available phosphorus. Dahlgren (6) modified the procedure by replacing HCl with HNO_3 and by adding citric acid and acetone. He named the solution quimociac. In 1963, Hoffman and Wiles (7) successfully used this new acetone-bearing quimociac reagent for determining total and available phosphorus by the gravimetric procedure. The method, AOAC **2.026-2.028** (8), adopted as official final action in 1965 (9), has become one of the most popular methods for phosphorus determination. However, because of the amount of energy expended in drying ovens, an investigation was undertaken to determine the feasibility of using a microwave oven for drying the yellow quinolinium molybdophosphate precipitate. Subsequent research resulted in the modification described below.

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METHOD

Apparatus and Reagents

(a) *Microwave oven.*—Power settings that include 600 watts.

(b) *Drying oven.*—Capability of drying 30 min at 250°C.

(c) *Gooch crucible.*—50 mL, stemmed, high form, with medium porosity fritted disc.

(d) *Quimociac reagent.*—See **2.026(c)** (8).

Sample Preparation and Determination

According to **2.027**, weigh 1 g fertilizer sample into 200 mL volumetric flask (or Kjeldahl flask for samples high in organic matter). Add 20–30 mL HNO_3 and boil gently 30–45 min. Add 10–20 mL of 70–72% $HClO_4$ and boil very gently until solution is colorless or nearly so and dense white fumes appear in flask. Cool slightly, add 50 mL water and boil 3–4 min. Cool solution, fill to volume, mix, and let settle overnight (or filter through dry filter). According to **2.028**, pipet aliquot containing maximum 25 mg P_2O_5 into 500 mL Erlenmeyer flask and dilute to ca 100 mL with water. Add 50 mL quimociac reagent, cover with watch glass, place on hot plate in well ventilated hood, and boil 1 min. Cool to room temperature (swirl carefully 3–4 times during cooling) and filter into previously dried and weighed gooch crucible. Wash with five 25 mL portions of water. Place in plastic or other suitable rack and transfer to microwave oven. Do not put metal in microwave oven! Dry crucible and contents at 600 watts until weight loss is negligible (ca 30 min for set of 12). Cool in desiccator to room temperature and weigh. Multiply by 0.03207 to obtain weight of P_2O_5.

% P_2O_5 = (g yellow ppt × 200 mL × 0.03207 × 100)/(g sample × mL aliquot).

Results and Discussion

A study was conducted to determine the time required to dry the quinolinium molybdophosphate precipitate with a microwave oven at 600 watts power. As expected, the time varied according to quantity of precipitate in each gooch crucible and total number of crucibles with precipitate. In general, ca 30 min was required to

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Table 1. Comparison of 2.026–2.028 and microwave drying methods for determining P_2O_5 in collaborative fertilizer samples from Magruder

Sample	% P_2O_5, microwave[a]	% P_2O_5, AOAC[a]		
		Mean	SD	No. labs
7904	4.31	4.33	0.33	27
8007	7.58	7.47	0.06	34
7912	8.18	8.18	0.07	24
7908	10.06	10.11	0.07	29
8004	10.13	10.15	0.06	30
7903	10.79	10.77	0.14	31
8002	11.05	11.01	0.09	27
8005	14.14	14.13	0.21	30
7901	15.05	15.19	0.15	25
7902	17.20	17.07	0.11	27
7910B	17.72	17.79	0.08	30
7911	19.97	19.83	0.13	25
7905	27.64	27.62	0.21	25
7909	28.86	28.85	0.14	25
7907	34.09	34.17	0.32	29
8008	46.31	46.36	0.35	33
8003B	54.51	54.53	0.22	29

[a] Average of duplicates.
[b] From Magruder reports.

dry 12 crucibles with each containing 0.5 g precipitate. If there is any doubt concerning dryness after microwave drying, remove a representative gooch crucible and weigh immediately (may use 3-place top loading balance): dry 5 additional min and weigh again. If there is no weight loss after additional drying, crucibles and contents may be placed in desiccator according to described method. If yellow precipitate is thoroughly dry, the gooch crucible will be quite hot because there is little water to absorb the microwave energy.

In another study, reagent grade KH_2PO_4 containing 52.2% P_2O_5 was analyzed 33 times by the described procedure. The mean calculated for the 33 determinations was 52.2% P_2O_5 with a standard deviation of 0.2 and a coefficient of variation of 0.4%. These statistical results were similar to those found for KH_2PO_4 by **2.026-2.028.**

Seventeen Magruder check fertilizer samples were analyzed for P_2O_5 by the described method, and results were compared against means compiled from reports of participating laboratories (Table 1). There were no significant differences ($P < 0.05$, Student's t-test (10)) in P_2O_5 determined by the 2 methods.

To compare the 2 drying techniques in our laboratory, 8 fertilizer samples from the State control service were digested; equal aliquots were taken for precipitate formation and drying—one by microwave and the other by conventional drying oven. No significant differences ($P < 0.05$ (10)) for P_2O_5 were found by the 2 methods (Table 2). Over 4000 samples have been dried by microwave since this initial investigation; about 400 were re-analyzed by **2.026-2.028**. No practical differences have been found to negate the validity of the initial findings.

A distinct advantage of the described method of drying over **2.028** is that the yellow precipitate will not char with reasonably excessive drying. Many hours have been lost with the AOAC method due to charring; however, we have excessively dried for up to 15 min by the described method without significantly changing weight of the precipitate.

Much less energy is expended with the microwave oven than with the drying oven; with most laboratories having limited budgets, the monetary savings is welcomed. There is little or

Table 2. Comparison of 2.026–2.028 and microwave drying methods for determining P_2O_5 (%) in fertilizers received through State control service

Guar.	Microwave	AOAC
5.0	3.4	3.5
5.0	5.6	5.5
10.0	6.9	6.8
10.0	8.3	8.2
12.0	24.0	24.1
25.0	27.3	27.2
32.0	34.6	34.7
46.0	45.9	45.9

no savings in time of actual drying; however, in the drying oven, time must be allowed to attain the 250°C that **2.028** requires. No warmup time is required for microwave drying. Also, cost for the 2 instruments is comparable. For example, a $450 microwave oven has been used in our laboratory for ca 700 h with no major repairs required.

In addition to drying the quinolinium molybdophosphate precipitate for P_2O_5 determination in fertilizers, we are discovering additional uses for the microwave oven in our laboratory. These findings will be related as soon as the data have been compiled and tabulated.

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Data Handler/Controller System with Application to P_2O_5 and K_2O in Fertilizers

PETER F. KANE, BRIAN R. BENNETT, and STEFAN GULIK
Purdue University, Department of Biochemistry, West Lafayette, IN 47907

An automated data handler/controller system performs 2 main functions: As a data handler, it finds peak maxima, does standard curve fitting, performs peak height correction for drift, gain, and carryover, and does limited calculation of results with a printed record. It may also control modules of an analyzer system to allow both manual operation and completely automatic, unattended startup, operation, and shutdown. Such a system has 2 main advantages, and one disadvantage. The automatic control features extend the useful operational hours, and the data handling features make possible a level of sophistication above manual treatment of the data, with no computer expertise required. To achieve these ends, however, the flexibility that comes with control over software modification has been sacrificed. The ability to correct peak heights for drift, gain, and, particularly, carryover, allows a wider range of standards and/or faster sampling rates. In the phosphorus method, it was possible to eliminate much labor-intensive aliquoting of extracted samples by using a broader range of standards. Alternatively, if desired, the sampling rate could be increased from 40 to 60 per hour. With computer-corrected peak heights, either alternative is still more precise than the usual method without correction. For the potassium method, the maximum concentration is limited by the detector of the flame photometer and therefore the standard range cannot be significantly expanded. However, dramatic increases in sampling rate are possible with peak height correction. Data indicate that sampling rates can be increased from the usual 40-50 per hour up to 90 or possibly 120 per hour, with equivalent precision.

The Indiana State Chemist Laboratory recently had the opportunity to evaluate a Technicon AAIIC data handler/controller system made available to us from Technicon. The AAIIC is designed to operate with AutoAnalyzer II Systems. It performs 2 main functions: data manipulation and automatic instrument control. These 2 functions will be examined first, and then specific application to the AOAC automated P_2O_5 and K_2O methods for fertilizers will be discussed.

Data Handler

The data handling features fall between manipulating automated analyzer chart output with a programmable calculator, and having analyzers on-line to a large computer system. The data handling capabilities include finding peak maxima, standard curve fitting, peak height adjustment features such as carryover, drift, and gain correction, and final calculation with production of a printed record of results.

Curve fitting options include linear, second degree, and piecewise linear. Baseline is automatically included as one of the points on the curve to be fitted. Calculations can be redone on the same data with any of the curve fitting options.

Peak height correction capability is one of the more attractive features. Drift is detected by monitoring the baseline for stability before a run is started. A stable value is stored. When sampling is completed, the baseline is again monitored and peak heights are incrementally corrected for any drift.

Any change in gain is detected by placing a designated high standard near the beginning and ending of a run. The effect of this correction is to incrementally rotate the standard curve best fitted line as it is compared with each sample peak height for calculation.

The last peak correction feature is carryover. Many automated methods have a carryover effect from one sample to the next. Often this is an important limiting factor, restricting the maximum sampling rate and the standard range for a method. For a given chemistry and a specific manifold, the carryover factor is relatively constant and easy to determine, but the arithmetic required for manual carryover correction of peak heights is quite tedious and time-consuming. Traditionally, the way of dealing with carryover has been to slow the sampling rate and narrow the standard range until any remaining carryover is insignificant relative to the degree of precision required of the method. The data handler allows computer-calculated peak height correction for carryover. Data will be presented illustrating the improvement in precision and/or speed which can be attained in the specific instances of the P_2O_5 and K_2O methods.

As a test of how well the data handler finds accurate peak heights, it was connected to a Flame IV system used for fertilizer K_2O. This system is inherently more noisy than colori-

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Table 1. Determination of peak maxima, K_2O oxalate standards [a]

AAIIC peak maxima	Computer peak maxima	Normalized computer peaks	AAIIC minus computer normalized
5.22	5.225	5.231	−0.011
20.2	20.075	20.098	0.102
35.1	35.125	35.165	−0.065
50.1	50.04	50.097	0.003
65.8	65.75	65.825	−0.025
81.6	81.55	81.644	−0.044
89.6	89.705	89.808	−0.208
5.25	5.12	5.126	0.124
4.99	4.90	4.906	0.084
20.1	19.96	19.983	0.117
20.1	19.96	19.983	0.117
35.2	35.125	35.165	0.035
35.4	35.295	35.335	0.065
50.3	50.225	50.283	0.017
50.3	50.245	50.301	−0.003
65.8	65.75	65.825	−0.025
65.9	65.865	65.941	−0.041
81.8	81.75	81.844	−0.044
81.6	81.72	81.814	−0.214
89.4	89.415	89.518	−0.118
89.6	89.365	89.467	0.133
$\bar{X}$ = 49.6838	$\bar{X}$ = 49.6269	$\bar{X}$ = 49.6838	$\bar{X}$ = 0.0000 SD = 0.099

[a] Peak heights in chart divisions.

metric systems, and therefore is a good test for finding peak maxima.

Peak heights are not displayed directly, only calculated concentrations relative to standard concentrations. However, it is possible to input a standard's peak height rather than its concentration, thus forcing a display of sample peak heights instead of concentrations.

Table 1 compares the AAIIC peak heights for a series of K_2O standards with peak heights from the same run determined by an on-line computer system routinely used by this laboratory. This latter system consists of an A to D converter, a Digital PDP 11/15 with software to determine and store peak heights, and a PDP 11/45 which receives peak height files from the PDP 11/15 and performs all additional calculations. Computer-plotted graphs of peak heights vs standard concentrations were virtually identical for the 2 sets of peak height data.

Technically, the data handler peak heights are all determined relative to one PDP 11/15 peak height; therefore, it is reasonable to normalize one entire set of peaks to the other, based on overall means. Arbitrarily normalizing the computer-plotted peaks to the data handler peaks so that the difference of the means of the 2 sets is reduced to zero chart divisions, the question then is, how consistently small are the individual differences? The standard deviation of the differences is ±0.099 chart division. In only 2 instances does a difference exceed 2 standard deviations. This surely exceeds the precision to be expected from manually determining peak heights.

The data handler has a limited calculating function. Knowing the standard and sample peaks heights, it automatically produces a sample concentration as analyzed by the analyzer. This concentration can be recalculated by any combination of the curve fitting and data manipulation options. However, the data handler has no capability to store factors for aliquots, sample weights, stoichiometry, etc. It is necessary to type in a single overall factor for each sample in turn for the system to calculate final results.

In addition to producing a printed record of final results, several other features that may be useful in various applications are error messages with peak heights, user specification of baseline noise tolerance, ability to flag upper and lower tolerance limits, and statistical routine for samples and intersample standards. The maximum number of standards in a run is 8. The maximum number of total cups in a run varies from 160 for a single-channel operation to 40 for 4-channel operation. Calculations on a run must be completed before another run is begun.

Instrument Controller

In addition to manipulating data, the system serves as an instrument controller. In either programmed or manual modes, it will control a sampler, a pump, an Auto Valve® switching between reagent and wash solutions, and several auxiliary electrical outlets. The sampling rate and sample-to-wash ratio, no longer controlled by a mechanical cam, may be set to any desired rate and ratio.

To determine if computer-controlled sampler timing is significantly more precise than timing by mechanical cam, multiple cups of high standard were analyzed 40 times by both instruments on the P_2O_5 analyzer. Peak heights vs peak positions were fitted to a least squares straight line to correct for drift. For the computer timed run, the mean corrected peak height was 88.3188 chart divisions with a standard deviation of 0.119. For the cam-controlled run, the mean corrected peak height was 86.5315 chart divisions, with a standard deviation of 0.121. The standard deviations are virtually identical, indicating similar precision between methods of timing, at least with the particular cam used, and for the P_2O_5 method. It may be that other methods, operating farther

from steady state conditions, would show more of an improvement in precision with computer-controlled timing. However, this has not been investigated.

The pump may be controlled in either a continuous or an intermittent mode. In the intermittent mode, the pump runs about 1 s every 2-3 min. This prevents the pump tubes from being crimped, allows for an automatic shutdown, or allows the system to be maintained in a near ready state with negligible reagent consumption. Pump tubes last several weeks when kept under continuous platen pressure.

The Auto Valve® is installed between reagent reservoirs and analyzer manifold with transmission tubing. An additional line to a wash reservoir allows the valve to switch all the reagent lines to this wash solution simultaneously. In addition to main reagent valves, there is an independently controlled auxiliary valve. For P_2O_5 analysis, this valve is used to allow an initial dilute HCl wash of the system, followed by the regular water wash. Dilute HCl reduces drift. In another application, the auxiliary valve is used for one of the reagents which needs to be added a few minutes after the others.

By using the various programming options, an analyzer can be automatically controlled after hours, cycling through an extended shut-down sequence, then switching to intermittent mode.

It is our experience that sample evaporation becomes significant if samples remain in uncovered cups much more than 1 h. A simple system can be devised from sample cup caps with holes drilled in them, PVC food wrapping, and a non-coring hypodermic needle in place of the sample probe.

Advantages and Disadvantages

In the authors' laboratory, analyzers are normally on-line to the previously described computer system. Several computer personnel on staff maintain and develop the system. When discussing advantages and disadvantages of an automated data handler, we have a different perspective compared with the laboratory with one or two analyzers, graph paper, and a calculator.

In general, the main advantage of an automated data handler is that it can be set up quickly by someone with no knowledge of computers. The user can do calculations and manipulations of data which would be either tedious or wholly impractical to do manually. In addition, the instrument control features significantly extend the length of time analyzers can be operating.

The biggest disadvantage is inflexibility. Programming is not modifiable without changing hardware. As an example, to gain precision a number of AOAC automated methods are designed with a narrow standard range, with baseline off scale. However, the AAIIC requires that the baseline be one of the calibration points on the standard curve. It also uses on-scale baseline to determine both drift and stability before beginning runs. It is possible to have the baseline off scale and get usable data, but one must use piecewise linear standard fitting, disregard results between zero and low standard, and also sacrifice the drift correction and initial stability checking features.

A second limitation is data storage capability. Runs need to be shorter than we are accustomed to, especially for multichannel systems. Also it is necessary to completely calculate one run before another is begun. If the calculation factor is different for each sample and a function of several variables, as it is for all our systems, then that composite factor must be hand-calculated for each sample and entered into the data system one sample at a time.

The basic trade-off is ease of installation and maintenance of programming on the one hand and the flexibility to tailor software to a laboratory's specific needs on the other. A laboratory should evaluate its overall computer goals and needs in this light.

Application to P_2O_5 Determination in Fertilizers

Because greater precision is expected with carryover and other peak height corrections, we hoped that the standard range in the direct available automated P_2O_5 method, AOAC **2.032** (1), could be expanded enough to eliminate all aliquoting of the citrate extract. This would require a standard range of 0-1.2 mg P_2O_5/mL compared with range of 0.15-0.35 mg/mL in the official method. Standards of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mg P_2O_5/mL were prepared, and analyzed at a sampling rate of 40/h, 2:1 sample-to-wash ratio on the automated instrumentation specified by **2.032**. The standard curve was quite nonlinear, and the 0.1 mg/mL standard was obscured as a shoulder on a preceding 1.2 mg/mL peak. Reducing the sampling rate to 30/h separated the 0.1 mg/mL peak, but even a fifth degree fit of the standard peak heights was not

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Table 2. P_2O_5 results (%) calculated by data handler and by computer

Material	AAIIC[a]	Computer[b]	Magruder grand avs[c]
NBS $NH_4H_2PO_4$	61.8	62.15	61.684
	61.7	61.73	—
	61.9	61.81	—
NBS KH_2PO_4	52.3	52.24	52.105
	52.2	52.20	—
	52.2	52.14	—
Mag. 7901B	14.7	14.70	14.597
7902	17.0	17.04	17.201
7903	10.1	10.22	10.735
7904	4.35	4.41	4.574
7905A	26.7	26.83	26.819
7907B	33.9	33.97	34.079
7908	9.78	9.67	9.856
7910B	17.1	17.16	17.319

Av. bias, Magruder G. A. minus AAIIC = +0.07
Av. bias, Magruder G. A. minus computer = +0.02
SD of diffs, Magruder G. A. minus AAIIC = 0.232
SD of diffs, Magruder G. A. minus computer = 0.225

[a] Nonlinear, drift, gain, and carryover corrected.
[b] Third degree, drift, gain, and carryover corrected.
[c] From Magruder reports.

good. (This laboratory can computer-generate any degree fit of a data set, and computer-plot the results. Various degree fits can be superimposed and visually compared.)

We decided to reduce the standard range to 0–0.8 mg P_2O_5/mL which would eliminate pipetting for fertilizers with 40% P_2O_5 or less, the bulk of this laboratory's sample load. All higher guarantees would be diluted 50 mL to 100 mL. Standards of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 mg P_2O_5/mL were run at a sampling rate of 30/h. A third degree curve adequately fit the peak height vs concentration data, while a second degree fit was less desirable. It is possible to reduce the number of calibration points by 4, recalculate another third degree fit of the remaining points, and have this line be virtually superimposable on the third degree line from the complete set of points. Thus 8 calibration points are more than adequate to define the curve.

The analytical test cartridge was modified to reduce the PO_4^{-3} concentration at the flowcell, straightening the standard curve somewhat. The sample pump tube was changed to 0.16 mL/min, the resample was changed to 0.16 mL/min, the $HClO_4$ pump tube was changed to 0.8 mL/min, and the sampling rate was 30/h, 2:1 sample-to-wash ratio. All other parameters were the same as specified in the official AOAC method. Any smaller sample-to-reagent ratio led to excessive noise. With these parameters, the standard peak heights still were fitted better by a third degree rather than second degree equation. A flowcell with a shorter path length, if available, would probably lessen the degree of curvature further.

A set of NBS Standard Materials and Magruder samples were analyzed using the above parameters. Results were calculated using the AAIIC and this laboratory's PDP 11/15–PDP 11/45. The AAIIC calculation used nonlinear (second degree) curve fitting with carryover, drift, and gain corrections. The PDP calculation used third degree curve fitting. The PDP software includes programming which also allows carryover, drift, and gain corrections. These calculations are analogous to the corresponding calculations performed by the AAIIC, but slightly more elegant.

Table 2 gives the results for a simultaneous calculation of the same analyzer run by both systems. The Magruder grand averages for direct available P_2O_5 by the AOAC automated method and the NBS theoretical values are given for comparison. Bias and standard deviation of differences are all acceptable. Results indicate that a modified P_2O_5 method with expanded standard range and much reduced aliquoting is feasible with computer peak height correction. Also, there is basic agreement between AAIIC and PDP computer calculations. The modified method would mean a significant savings in analyst time, elimination of some repeat analyses due to wrong aliquot prediction and off-scale peaks, and elimination of some human error associated with the aliquoting process.

This laboratory is primarily interested in precision and reduction of aliquoting. Sampling rate is less of a consideration, especially when the capability exists to automatically control analyzers after hours. However, carryover, drift, and gain correction of peak height data can be used to increase the sampling rate of an analyzer, if that is the main priority.

The six standards of the AOAC official method, 0.15–0.35 mg P_2O_5/mL, were analyzed in random order in a set of 40. The same order of analysis was repeated at rates of 40/h, 50/h, and 60/h. The raw peak heights were corrected for drift, gain, and carryover with the computer system. The means and standard deviations of the peak heights of each standard, with and without corrections, are given in Table 3.

In every instance except standard No. 5 at 50/h, the standard deviations are improved with correction. The most dramatic improvements are seen at the lower 3 standard levels. At 60/h

Table 3. Effect of sampling rate on peak height (PH) precision for P_2O_5

P_2O_5 Standard	40/h, no correction	50/h, no correction	60/h, no correction	40/h, correction	50/h, correction	60/h, correction
0.15 mg/mL						
Mean PH	10.61	11.27	10.24	9.81	9.46	7.46
SD	0.502	1.014	1.772	0.153	0.153	0.168
0.19 mg/mL						
Mean PH	26.55	26.62	24.82	25.70	25.09	22.18
SD	0.406	0.886	1.447	0.069	0.129	0.117
0.23 mg/mL						
Mean PH	42.42	41.72	38.70	41.77	40.60	37.31
SD	0.354	0.672	0.278	0.118	0.297	0.270
0.27 mg/mL						
Mean PH	57.89	57.25	53.94	57.08	55.71	51.65
SD	0.223	0.370	0.918	0.081	0.296	0.421
0.31 mg/mL						
Mean PH	73.78	72.37	68.96	72.99	70.94	66.36
SD	0.406	0.298	0.452	0.377	0.318	0.387
0.35 mg/mL						
Mean PH	89.48	87.73	84.13	88.55	86.10	81.13
SD	0.383	1.241	1.220	0.335	0.422	0.187

without correction, the carryover can be as much as 3 to 4 chart divisions. But the corrected peak heights at 60/h are more precise than the uncorrected peaks at 40/h, the sampling rate in the official method, with the exception of standard No. 4. However, standard No. 4 at 40/h seems artificially precise compared with the precision of the other standards at 40/h, probably a chance circumstance. It is reasonable to expect that the P_2O_5 method could be set up to run at 60/h with correction, and still perform more precisely than the official method at 40/h without correction. If one wished to use the official method without change, it is still possible to gain increased precision at 40/h. In each instance in Table 3 at 40/h, precision is improved with correction, especially in the area of the lower peak heights.

To further demonstrate this improvement, 8 individual determinations were made of NBS $NH_4H_2PO_4$ (61.68% P_2O_5) according to the official method. The standard deviation of the calculated percent P_2O_5 was 0.147 without peak height correction, and 0.056 with correction. The peak heights all fell at about 50 chart divisions. Samples which aliquoted to higher peak heights would be expected to show less improvement in precision, while samples which aliquoted to lower peak heights would be expected to show even more improvement on the average.

In our practice, carryover is determined in each analyzer run. This requires only several extra sample cups per run. However, carryover should be consistent for a particular analyzer system, and it probably will be feasible to determine a carryover factor only periodically. More routine data need to be generated to verify this.

The carryover factor is given by $\exp^{-p/b}$, where p is the time interval between peaks and b is a constant (2). Because the carryover factors had been determined at different sampling rates for the P_2O_5 method, it was possible to determine b at the various rates, to verify that it is a constant. Table 4 lists carryover factors and the corresponding b values. The evident predictability of b for P_2O_5 lends weight to the idea that carryover will also remain reasonably constant at a given sampling rate over longer periods. The data handler system allows the option of determining carryover in each run, or using a known value predetermined in a special run.

Application to K_2O in Fertilizers

The physical connections of the data handler system to a flame photometer are not as straightforward as connection to a colorimeter system. The photometer does not have any direct output for a computer, so peak heights are monitored by means of a following potentiometer on the chart recorder. We have used a custom-built unit to bring the flame photometer

Table 4. Carryover factors

Sampling rate	Carryover factor	b
40/h	0.01143	0.0056
50/h	0.02648	0.0055
55/h	0.03544	0.0055
60/h	0.04786	0.0055

Table 5. Effect of sampling rate on peak height precision for K_2O

Rate	10 μg K_2O/mL Std		20 μg K_2O/mL Std	
	No carryover	Carryover	No carryover	Carryover
40/h, Mean PH	18.04	17.84	35.80	35.60
SD	0.151	0.089	0.141	0.163
50/h, Mean PH	18.10	17.92	37.75	35.53
SD	0.158	0.084	0.129	0.150
60/h, Mean PH	17.78	17.42	35.08	34.68
SD	0.311	0.084	0.150	0.126
72/h, Mean PH	18.06	17.52	35.28	34.75
SD	0.422	0.045	0.206	0.058
90/h, Mean PH	18.28	17.50	35.50	34.68
SD	0.669	0.071	0.216	0.096
120/h, Mean PH	18.74	17.42	36.10	34.80
SD	1.050	0.084	0.829	0.566
	30 μg K_2O/mL Std		40 μg K_2O/mL Std	
40/h, Mean PH	53.33	53.33	71.97	72.41
SD	0.379	0.351	0.058	0.140
50/h, Mean PH	53.23	53.23	71.77	70.76
SD	0.252	0.116	0.322	0.191
60/h, Mean PH	51.90	51.90	70.10	70.09
SD	0.265	0.000	0.173	0.078
72/h, Mean PH	51.90	51.87	69.73	69.69
SD	0.361	0.058	0.503	0.044
90/h, Mean PH	51.80	51.70	69.53	67.74
SD	0.557	0.100	0.862	0.363
120 h, Mean PH	51.57	52.43	68.70	60.66
SD	1.172	0.153	1.179	0.119
	50 μg K_2O/mL Std		55 μg K_2O/mL Std	
40/h, Mean PH	90.30	90.43	99.40	99.54
SD	0.337	0.330	0.255	0.219
50/h, Mean PK	89.93	90.03	99.18	99.32
SD	0.532	0.479	0.545	0.554
60/h, Mean PH	87.83	88.00	96.72	96.96
SD	0.435	0.383	0.259	0.152
72/h, Mean PH	87.83	88.10	96.68	97.00
SD	0.150	0.216	0.327	0.173
90/h, Mean PH	87.33	87.68	96.14	96.58
SD	0.263	0.096	0.434	0.228
120/h, Mean PH	86.45	87.13	96.86	96.64
SD	0.507	0.236	1.293	0.573

on-line to the computer. The same unit was used for connection to the AAIIC, although following potentiometers are available commercially.

There is a second problem in connecting to a flame photometer if automatic shutdown is desired. One of the auxillary electrical outlets of the Auto Valve could be used to shut off electrical power to the photometer. This would shut off the methane supply at the instrument, but would leave gas pressure in the supply lines. For safety reasons, an electrical solenoid shutoff at the methane tank valve would be desirable. A second such solenoid at the air supply valve might also be desired.

As with P_2O_5, we hoped that the standard range in the official automated flame photometric method for K_2O fertilizers, AOAC **2.097** (1), could be expanded sufficiently to eliminate aliquoting. This was not feasible. The potassium concentrations required by the specified 1 g sample weight badly saturated the potassium detector. Attempts at reducing sampling rate and increasing diluents on the manifold, and/or including a dilution loop in the manifold design, resulted in excessive noise well before the desired reduction in potassium concentration was reached. While it is possible to expand the standard range somewhat, this increase would have no effect on reducing the analyst workload when aliquoting the original sample extract.

Our photometer required slight adjustment of some of the manifold parameters specified in the AOAC method. Data presented refer to this adjusted procedure. Because expanded range

and reduced aliquoting did not prove feasible, attention was directed towards improving sampling rate and precision.

To bring the baseline on scale so that the data handler could be fully utilized, the low and high standards were adjusted to about 18 and 98 chart divisions, respectively, rather than the usual 10 and 90 chart divisions. With all other parameters remaining the same, a randomly ordered set of 40 standards was analyzed at sampling rates of 40/h to 120/h. Table 5 tabulates peak height means and standard deviations for each standard concentration. Our computer system gives similar results.

In analyzing the data in Table 5 it should be remembered that, because it is a flame rather than a colorimetric method, the chart tracing is more noisy. This would make it desirable to have multiple sets of carryover peaks so that a more accurate carryover factor could be calculated. At the time this work was done, the data handler programming limited multiple carryover determinations, but a newer version allows carryover determinations to be run separately before actual sample determinations. At 40/h the system operates with virtually no carryover. However, at 40/h the system calculated a carryover factor larger than that at 50/h, no doubt due to system noise on the carryover peaks. This would explain why at 40/h there are several instances where the precision is slightly greater without carryover correction than with correction.

A second factor to remember is that, because of noise, there are instances where precision seems worse at a low sampling rate, and better at a much higher sampling rate for the same standard. But what must be looked at are the general trends.

Without carryover correction, precision is about equivalent at sampling rates of 40, 50, and probably 60/h. Above 60/h, precision rapidly deteriorates. With carryover correction, precision is about as good even at 120/h as it is at 40/h, with the possible exceptions of standards No. 2 and 6. A larger volume of data would need to be generated to determine if these effects at 120/h were due to real loss of precision, or just the random effect of noise.

At 120/h, the system is operating 5 to 10 chart divisions away from steady state. A low peak is almost obscured as a shoulder on a preceding high peak. Table 5 demonstrates that there is considerable leeway for mathematically cleaning up such poor quality data, which would be totally unacceptable without correction.

Conclusion

Data calculation and instrument control provide a much needed link between analyzer and computer, expanding their capabilities. In comparison with a large, multipurpose laboratory computer system which combines a number of on-line instrumentation elements, administrative reporting elements, and personnel to maintain and modify software, the data handler does sacrifice flexibility. On the other hand, it requries no computer expertise to make it operational, it is available for immediate use, and the expense is in the range of the annual salary of one computer person. A laboratory would do well to evaluate these features in the context of its own long-range computer goals.

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OILS AND FATS

Chromatographic Separation of Polar and Nonpolar Components of Frying Fats

ARTHUR E. WALTKING[1] and H. WESSELS[2]
Best Foods, Research and Engineering Center, CPC International, Inc., Union, NJ 07083

A method is proposed to assess deterioration of frying fats by measuring polar and nonpolar components separated on a silica gel column. Means for polar components measured in duplicate samples by 19 collaborators ranged from 8.0 ± 0.34 to 25.8 ± 0.90%. Coefficients of variation ranged from 3.5 to 4.9%. The method has been adopted as official first action.

In a short report by the Associate Referee on Oxidized Oils at the 94th Annual Meeting of AOAC, Washington, DC, October 22, 1980, it was proposed that the method of Guhr and Waibel (G. Guhr & J. Waibel (1978) *Fette Seifen Anstrichm.* 80, 106–113) as proposed by Sen Gupta and Guhr be adopted official first action. This method has been extensively collaboratively studied by the IUPAC Commission of Oils, Fats and Derivatives Working Group No. 7. On the basis of their collaborative study (in which the AOAC Associate Referee participated), the IUPAC Commission has recommended the following procedure for adoption as an official IUPAC procedure:

Polar Components in Frying Fats – Official First Action

IUPAC-AOAC Method

Principle

Method assesses deterioration of used frying fats, and is applicable to all fats and oils. Polar components are those components of fats detd by column chromatgy under conditions specified, and include polar substances such as monoglycerides, diglycerides, free fatty acids that occur in unused fats, as well as polar transformation products formed during frying of foodstuffs and/or during heating. Nonpolar components are mostly unaltered triglycerides. Frying fats are sepd by column chromatgy on silica gel into nonpolar and polar components. Polar components are detd indirectly by subtracting concn of nonpolar components. Quality of sepn can be checked by thin layer chromatgy.

Apparatus

(**a**) *Column.*—Glass, 2.1 cm id × 45 cm, with Teflon stopcock and ground-glass joint.

(**b**) *TLC plates.*—Pre-coated silica gel (without fluorescence indicator), 20 × 20 cm, layer thickness = 0.25 mm.

Reagents

(**a**) *Adsorbent.*—Silica gel 60, particle size 0.063–0.200 mm (70–230 mesh ASTM), Merck No. 7734, or equiv., adjust to H_2O content of 5% as follows: Dry silica gel ≥4 h in porcelain dish in 160° oven; cool in desiccator to room temp. Adjust H_2O content to 5%, e.g., weigh 152 g silica gel and 8 g H_2O in 500 mL r-b flask with ground-glass stopper and mech. shake 1 h.

(**b**) *Eluting solvent mixture.*—Light petroleum (bp 40–60°)-ethyl ether (87 + 13).

(**c**) *Sea-sand.*—Anal. reagent grade; purified by acid and calcined.

(**d**) *Spray reagent.*—Molybdophosphoric acid, 10% in alcohol.

Preparation of Sample

Warm semi-liq. and solid samples to temp. slightly above mp and mix thoroly; avoid overheating. Remove visible impurities by filtration; if H_2O is present, use hydrophobic filter.

Preparation of Column

Fill column with ca 30 mL light petroleum–ethyl ether (87 + 13). Place wad of cotton wool in bottom of column and remove air by pressing with glass rod.

In 100 mL glass beaker, prep. slurry of 25 g silica gel and ca 80 mL light petroleum-ethyl ether (87 + 13) and pour slurry into column thru 8 cm glass funnel. Rinse beaker, funnel, and

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[1] A. E. Waltking is Associate Referee on Oxidized Oils.
[2] Chairman, IUPAC Commission of Oils, Fats, and Derivatives Working Group No. 7. Federal Center for Lipid Research, D-4400, Munster, Federal Republic of Germany.

The recommendation of the Associate Referee was approved by the General Referee and Committee C and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 65, March issue (1982).
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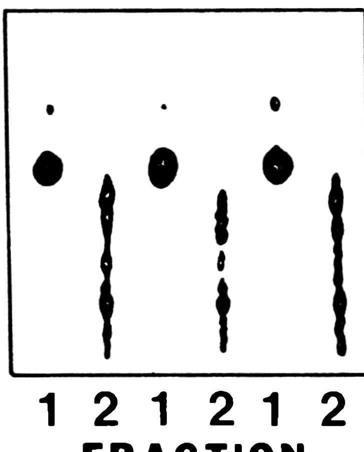

Figure 1. Evaluation of efficiency of fractionation by TLC separation of polar and nonpolar fraction; Fraction 1 contains nonpolar components, Fraction 2 contains polar components.

sides of column with same solv. Open stopcock and drain solv. to 10 cm above silica gel. Level silica gel by tapping column.

Add ca 4 g sea-sand thru funnel into column. Drain solv. to sand layer.

Chromatography

To det. polar components by diff., only nonpolar fraction is used. However, if sepn is controlled by TLC, both polar and nonpolar fractions are required. Sepn may also be controlled by checking recovery of sample, but for samples contg substantial amts of polar material, recovery may be incomplete because small amts of highly polar material, generally 1-2%, are not eluted under conditions specified.

Accurately weigh 2.5 ± 0.1 g (to 0.001 g) sample into 50 mL vol. flask, and dissolve in ca 20 mL light petroleum-ethyl ether (87 + 13) while warming slightly. Let cool to room temp. and dil. to vol. with same solv. Using vol. pipet, transfer 20 mL sample to column, without disturbing surface.

Dry two 250 mL r-b flasks in 103 ± 2° oven, cool to room temp., and accurately weigh to 0.001 g. Place one flask under column, open stopcock, and let sample soln drain to level of sand layer. Elute nonpolar components with 150 mL light petroleum-ethyl ether (87 + 13) contained in 250 mL dropping funnel. Adjust flow rate so that 150 mL passes thru column within 60-70 min. After elution, wash any substance adhering to outlet of column into r-b flask with light petroleum-ethyl ether (87 + 13).

Table 1. Statistical results of IUPAC collaborative study of polar components in frying fats

Sample	Mean[a], % polar components	SD	CV, %
1	8.0	0.34	4.3
2	7.3	0.36	4.9
3	11.5	0.55	4.8
4	25.8	0.90	3.5

[a] Samples analyzed in duplicate, 19 collaborators.

Elute polar components into second 250 mL r-b flask with 150 mL same solv. Discard silica gel.

Remove solv. from both fractions with a rotary evaporator and 60° water bath or with N stream in 250 mL beaker on steam plate. Avoid losses due to foaming. If rotary evaporator is used, shortly before end of evapn, introduce N into system from rubber bulb. Cool residue to ambient temp. and introduce N into flask. Weigh flasks.

Calculations

Calc. polar components, as % (w/w) by formula:

$$\text{Polar components, \%} = [(E - A)/E] \times 100$$

where A = g nonpolar fraction; E = g sample dissolved in 20 mL. Report result to 1 decimal place.

Control by Thin Layer Chromatography

Dil. polar and nonpolar fraction (1 + 9) in $CHCl_3$. Apply 2 μL spots using capillary dispensing pipet. Develop plate with light petroleum-ethyl ether-acetic acid (70 + 30 + 2) in tank lined with filter paper. Develop plate ca 35 min (ca 17 cm). Remove plate and let solv. evap.

Spray plate with 10% molybdophosphoric acid. After evapn of alcohol, heat plate in 120-130° drying oven.

Results

Figure 1 shows a chromatogram obtained after fractionation of a frying fat.

Key statistical elements from the IUPAC collaborative studies supporting the validity of this procedure are shown in Table 1. Complete details of the collaborative studies involving this methodology will be available in a future IUPAC publication. It is recommended that the method be adopted official first action.

Systematic Identification of Antioxidants in Lards, Shortenings, and Vegetable Oils by Thin Layer Chromatography

CARLOS H. VAN PETEGHEM, and DIANA A. DEKEYSER
Laboratorium voor Bromatologie, Faculteit Farmaceutische Wetenschappen, De Pintelaan 135, B-9000 Gent, Belgium

A simple and reliable method is described for rapid identification of ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, ethoxyquin, gallates (lauryl, octyl, propyl), nordihydroguaiaretic acid, 3,3′-thiodipropionic acid, tocopherol, *t*-butylhydroquinone, and 2,4,5-trihydroxybutyrophenone in lards, shortenings, and vegetable oils. The antioxidants are extracted with 95% methanol, concentrated under vacuum at ≤45°C, and analyzed by thin layer chromatography. Three elution solvents, 2 adsorbent types, 2 visualization sprays, and UV viewing at 254 and 366 nm are used. Sunflower and corn oil samples, fortified with 100 ppm antioxidant, were analyzed to establish the validity of the method.

Antioxidants are widely used to prevent oxidative degradation of fats, oils, shortenings, high-fat food, and certain food types such as potato flakes and granules, chewing gum, and flavoring extracts. Flavor stability is considerably enhanced by their action. In Belgium, the number of legal antioxidants is restricted compared with those permitted in the United States. Besides the naturally occurring tocopherols, only gallate esters (propyl, octyl, lauryl), BHT (3,5-di-tert-butyl-4-hydroxytoluene), and BHA (a mixture of 2- and 3-tert-butyl-4-hydroxyanisole) may be used. Single or combination maximum concentrations range from 100 mg/kg to 1 g/kg, depending on the type of food. In addition to these, the United States permits 2,4,5-trihydroxybutyrophenone (THBP), tert-butylhydroquinone (TBHQ), and 2,6-di-tert-butyl-4-hydroxymethylphenol in food for human consumption. Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) is used in animal feed for stabilizing β-carotene and vitamin A. It may also be used for the preservation of color in the production of chilipowder, paprika, and ground chili at levels not in excess of 100 ppm.

Other antioxidants which are encountered in other countries are nordihydroguaiaretic acid (NDGA) and 3,3′-thiodipropionic acid (TDPA). The antioxidant activity of browning products, obtained by reaction of low molecular carbonyl compounds with amino acids, is well known and has been intensively studied (1).

It was demonstrated that some compounds may act as synergists or antagonists when combined in food (2). Ascorbyl palmitate, like citric acid, is often classified as a pure synergist. Either it enhances the effect of a phenolic antioxidant or it forms complexes with traces of copper and iron and hence deactivates their prooxidant effect. Most of the antioxidants have been assigned toxicological no-effect levels and acceptable daily intakes (3–5).

Numerous methods have been published for qualitative and quantitative assay of antioxidants by means of colorimetry, thin layer chromatography, gas chromatography, and high performance liquid chromatography. A short review of the most interesting contributions is given by Kline et al. (6) and Page (7).

It is a problem to quantitatively isolate more or less polar compounds from a high-fat matrix and to obtain a final extract clean enough for final determination. Tedious extraction and cleanup procedures are common features of most existing methods. More polar compounds often need to be derivatized before gas chromatography. For example, the problem of low extraction efficiency and high volatility of BHT is well known to anyone involved in antioxidant analysis.

A second characteristic of existing methods is that they restrict themselves to a few substances. A new analytical procedure for identification of antioxidants should include as many as possible and should at the same time be rapid and reliable. In the method presented here, a simple extraction step with 95% methanol is used with a series of thin layer chromatographic systems to provide easy identification without any purification of the extract. The combination of eluting solvents and visualization reagents and methods enables one to establish the presence of 12 antioxidants with a high degree of probability. The method was successfully tried with vegetable oils, lards, and shortenings which had been fortified with normal amounts of antioxidants.

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METHOD

Apparatus

(a) *Rotary vacuum evaporator.*—Glass construction, spiral condenser, temperature-controlled water bath (Tokyo Rikakikai Co., Ltd.), or equivalent.

(b) *TLC plates.*—Silica gel plates, 20 × 20 cm, layer thickness 0.25 mm, with 254 nm fluorescence indicator (Merck No. 5715); polyamide plates, 20 × 20 cm, layer thickness 0.15 mm, with 254 nm fluorescence indicator (Merck No. 5557).

(c) *Spotting pipets.*—10 μL capacity with 1 μL graduations (Haak, or equivalent).

(d) *UV lamp.*—Excitation wavelengths 254 and 366 nm.

Reagents

All reagents used were analytical grade.

(a) *Methanol.*—95%.

(b) *Ethanol.*—96 proof, freshly distilled.

(c) *Antioxidants.*—Ascorbyl palmitate (AP), BHA, BHT, lauryl gallate (LG), octyl gallate (OG), propyl gallate (PG) from Fluka, Switzerland; ethoxyquin from J. Dekker, Naarden, The Netherlands; NDGA, TDPA, and TBHQ from Aldrich Europe, Belgium; DL-α-tocopherol from Merck, Germany; THBP from Sigma Chemical Co., St. Louis, MO.

(d) *Reference standard solutions for TLC.*—Accurately weigh and transfer 50 mg of each antioxidant (except 100 mg BHT and TDPA) into 10 mL volumetric flask, dissolve in 96° ethanol and dilute to volume. If screened from daylight and stored at 4°C, solutions are stable at least 4 weeks, except AP which has to be prepared every other day. Several reference standards may be combined in one solution to decrease number of solutions.

(e) *Standard solutions for control samples.*—Accurately weigh and transfer 0.500 g of each antioxidant into 100 mL volumetric flask, dissolve in acetone, and dilute to volume. Keep cool and screened from sunlight (see **d**).

(f) *Elution solvents.*—Solvent A: petroleum ether–benzene–acetic acid (40 + 40 + 20). Solvent B: *n*-hexane–acetone–acetic acid (55 + 40 + 15). Solvent C: methanol–acetone–water (60 + 20 + 20).

(g) *Reagent 1 (Emmerie-Engel reagent).*—Ferric chloride solution: Dissolve 100 mg ferric chloride in 100 mL distilled water. 2,2′-Bipyridin solution: Dissolve 500 mg 2,2′-bipyridin in 100 mL 96° ethanol.

(h) *Reagent 2.*—Dissolve 100 mg 2,6-dibromoquinone-4-chlorimide in 100 mL 96° ethanol.

(i) *Control samples.*—100 ppm. Accurately weigh 100 g sunflower oil containing no antioxidant except naturally occurring tocopherol in vacuum evaporator flask, add 2 mL standard solution for control samples, and mix thoroughly Evaporate solvent at ≤45°C at reduced pressure until no acetone odor is perceptible. Prepare control samples of same concentration in corn oil in same way. Keep screened from daylight.

Extraction Procedure

Weigh 10 g oil in 50 mL glass-stopper tube. Add 25 mL 95% methanol and shake vigorously ≥5 min. Centrifuge 10 min and decant alcohol layer. Repeat extraction with another 25 mL 95% methanol. Combine alcohol layers. Evaporate to 3-4 mL in rotary vacuum evaporator at ≤45°C. Transfer quantitatively to conical test tube and concentrate in nitrogen stream, carefully avoiding blowing extract dry. Reconstitute with 1 mL 96° ethanol.

Weigh 10 g lard or shortening, add 50 mL 96° ethanol, and reflux on boiling water bath 30 min. Cool and filter. Evaporate filtrate to 3–4 mL and continue as for liquid samples.

Thin Layer Chromatography

Apply 2 cm from the bottom and 1 cm apart 2 μL of each reference standard solution together with aliquot (2–8 μL) of sample extract on 2 silica gel plates. Dry spots in cold air stream. Develop one plate in elution solvent A, and the other in elution solvent B, both to 15 cm. Dry plates in cold air stream and observe under UV light. Thoroughly spray plate developed in solvent A with 2,6-dibromobenzoquinone-4-chlorimide reagent and heat 5 min at 105°C. While still hot, place plate in tank saturated with ammonia vapor. Spray the other plate thoroughly with ferric chloride solution, dry in cold air stream, and spray immediately with 2,2′-bipyridin reagent. After each step, carefully compare spot colors. Finally, observe under UV light.

If any doubt remains after examination of either plate, apply standards and unknown on polyamide plate and develop in solvent C. After elution, dry in cold air stream and visualize as for plate for solvent A.

Results and Discussion

Spiking of blank oil samples was more complicated than generally presumed. Not all an-

Table 1. Approximate R_f value of antioxidants separated with various thin layer chromatographic systems

Antioxidant	System A[a]	System B[b]	System C[c]
Ascorbyl palmitate	0.12	0.38	0.04
Butylated hydroxyanisole (BHA)	0.48	0.50	0.44
Butylated hydroxytoluene (BHT)	0.79	0.75	0.36
Ethoxyquin	0.22	0.60	0.59
Lauryl gallate (LG)	0.21	0.43	0.13
Nordihydroguaiaretic acid (NDGA)	0.06	0.34	0.25
Octyl gallate (OG)	0.17	0.41	0.25
Propyl gallate (PG)	0.09	0.32	0.45
3,3′-Thiodipropionic acid (TDPA)	0.25	0.26	—[d]
α-Tocopherol	0.61	0.70	0.03
t-Butylhydroquinone (TBHQ)	0.30	0.45	0.48
2,4,5-Trihydroxybutyrophenone (THBP)	0.19	0.41	0.37

[a] Silica gel plates; benzene–petroleum ether–glacial acetic acid (40 + 40 + 20).
[b] Silica gel plates; hexane–acetone–glacial acetic acid (55 + 40 + 5).
[c] Polyamide plates; methanol–acetone–water (60 + 20 + 20).
[d] Not detectable.

tioxidants were soluble enough in sunflower or corn oil to be dissolved by simple addition of the pure compound and subsequent thorough mixing. Addition of a concentrated ethanolic solution of the standards gave erroneous positive results upon extraction. When 2 mL ethanolic solution of any antioxidant was added to 100 g vegetable oil and the whole was thoroughly mixed, the compound could be recovered by simple mixing for 5 s with a small volume of 95% methanol, apparently because it had not been dissolved in the oil.

Therefore standard solutions were prepared in acetone, which is miscible with most oils. After careful mixing of 100 g oil and 2 mL standard solution, the solvent was evaporated in a rotary vacuum evaporator at ≤45°C until the pungent acetone odor was no longer perceptible. This usually took more than 30 min. Completely translucent oils were obtained with no visible particles in contrast to the presence of particles when the antioxidants were added as such to the oils.

Particular emphasis should be given to the extraction duration. An acceptable recovery was achieved when the solvent-oil mixture was vigorously shaken at least 5 min. The extraction precision was not determined because our purpose was identification only; evaluation was based on detectability of normally encountered or reasonable antioxidant concentrations (about 100 ppm). The chromatographic separation usually included silica gel plates, 2 elution systems, and 2 visualization methods. Addition of acetic acid minimized spot tailing. The combination of R_f values (Table 1) and spot color (Table 2) was a satisfactory means of identification for

Table 2. Color differentiation of antioxidants on thin layer chromatographic plates

Antioxidant	Reagent 1[a]	Reagent 2[b]
Ascorbyl palmitate	red	orange
Butylated hydroxyanisole	yellow	lilac
Butylated hydroxytoluene	red	yellow[c]
Ethoxyquin	red	yellow green
Lauryl gallate	bluish grey	light brown
Nordihydroguaiaretic acid	bluish grey	brown
Octyl gallate	bluish grey	light brown
Propyl gallate	bluish grey	light brown
3,3′-Thiodipropionic acid	—[d]	pale blue
α-Tocopherol	red	yellow
t-Butylhydroquinone	pink	violet
2,4,5-Trihydroxybutyrophenone	brown green	brown

[a] Ferric chloride-2,2′-bipyridin.
[b] 2,6-Dibromoquinone-6-chlorimide and ammonia.
[c] With blue fringe.
[d] Not detectable.

Table 3. Color differentiation of antioxidants after separation on polyamide layer and vaporization of reagent 2 (2,6-dibromoquinone-6-chlorimide and ammonia vapors)

Antioxidant	Reagent 2	Fluorescence, 366 nm
Ascorbyl palmitate	white[a]	yellow
Butylated hydroxyanisole	blue	—[b]
Butylated hydroxytoluene	white[a]	—[b]
Ethoxyquin	violet	blue
Lauryl gallate	yellow	yellow
Nordihydroguaiaretic acid	blue	orange
Octyl gallate	yellow	yellow
Propyl gallate	yellow	yellow
3,3′-Thiodipropionic acid	—[c]	—[b]
α-Tocopherol	white[a]	—[b]
t-Butylhydroquinone	pink	—[b]
2,4,5-Trihydroxybutyrophenone	yellow	—[b]

[a] Easily perceptible on a light brown background.
[b] Absorption.
[c] Not detectable.

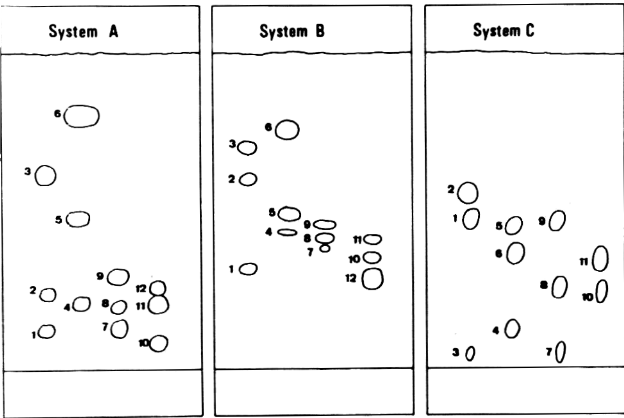

Figure 1. Tracing of typical TLC separations of antioxidants.

A, benzene/petroleum ether/glacial acetic acid (40/40/20) on silica gel plates; B, hexane/acetone/glacial acetic acid (55/40/5) on silica gel plates; C, methanol/acetone/water (60/20/20) on polyamide plates; 1, propyl gallate; 2, ethoxyquin; 3, α-tocopherol; 4, lauryl gallate; 5, butylated hydroxyanisole; 6, butylated hydroxytoluene; 7, ascorbyl palmitate; 8, octyl gallate; 9, *t*-butylhydroquinone; 10, nordihydroguaiaretic acid; 11, 2,4,5-trihydroxybutyrophenone; 12, 3,3′-thiodipropionic acid.

most antioxidants in most samples. Separations are illustrated on Figure 1.

If resolution was incomplete and color differentiation did not provide an unequivocal result, a third chromatogram on a polyamide layer was run. It was visualized with reagent 2 and UV viewing (Table 3). Several antioxidants showed a pronounced fluorescence on this type of adsorbent, especially ethoxyquin when irradiated with UV light of 366 nm. This fluorescence was much less on silica gel plates and of almost no practical value. AP, PG, and NDGA which were poorly resolved on silica gel with either eluant could easily be separated on the polyamide layer.

OG, THBP, LG, and ethoxyquin could not be differentiated with elution system A, nor, except for the latter, with elution system B, but elution system C provided a decisive answer. The same applied to PG and NDGA.

The presence of TDPA had to be ascertained after spraying the 2,6-dibromoquinone-4-chlorimide reagent and before saturation with ammonia vapors. When TDPA was presumed, another silica gel plate had to be developed but with elution system B, because polyamide adsorbent was unable to resolve the compound. Most likely TDPA moved with the solvent front on the polyamide. THBP as such showed an orange fluorescence when irradiated with UV light at 366 nm.

The intense blue fluorescence of ethoxyquin on polyamide plates was not affected by the application of spray reagent 1.

Color development of BHA and BHT spots with ferric chloride–2,2′-bipyridin reagent was rather slow. Sufficient time should therefore be allotted for the reaction to take place. Tocopherols and tocotrienols were not separable by the technique described; the compound DL-α-tocopherol was used as representative of that group of compounds, although γ- or δ-tocopherol may have been more appropriate with respect to relative antioxidant activity. These compounds may, however, be differentiated by other methods whenever required (8–10).

The detection of α-tocopherol on silica gel plates after elution with solvent system B was sometimes seriously impeded by the presence of interfering substances co-extracted from the fatty medium. They yielded light brown to yellow spots with either visualization reagent and were easily localized under 366 nm UV light. Here, too, the polyamide plate was able to provide a decisive answer if there was any doubt.

The technique of spraying was quite important. Because not all antioxidants showed the same high reactivity toward the color reagents, extreme attention had to be paid to an even application of solutions. Before visualization, acetic acid from elution systems A and B had to be completely removed in a cold air stream so as not to disturb the color development. Residues influenced not only color development but also

color shade. Therefore, one should not attach a great importance to the shades mentioned in Tables 2 and 3.

Shades were not entirely reproducible, so colors should be compared with reference standards. Spraying reagents were prepared fresh before use, and the concentrated ammonia solution had to be renewed after each application.

The limits of measurements, under the conditions described, were in the ppm range, except for AP, BHT, TDPA, and α-tocopherol which were about 50 ppm.

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VITAMINS AND OTHER NUTRIENTS

Evaluation of Ion Exchange Resins and Various Enzymes in Thiamine Analysis

WAYNE C. ELLEFSON, EARL RICHTER, MARK ADAMS, and N. THOMAS BAILLIES
Raltech Scientific Services, 3301 Kinsman Blvd, Madison, WI 53707

Four ion exchange resins and 9 enzyme preparations are evaluated for use in the official AOAC thiamine method because Decalso and Clarase or Mylase P either are no longer available or are available in a form that is not suitable for use in the assay. The enzymes are prepared in the same manner described for Clarase or Mylase P in the AOAC method and are compared with Clarase T300 for their effectiveness in releasing thiamine from thiamine phosphate, and their ability to produce similar results on samples. Rhozyme S is 90–100% as effective as Clarase T300 in both of these respects. The other enzymes tested were not satisfactory. Further study is necessary because Rhozyme S also is no longer manufactured. The ion exchange resins are prepared for use in the manner described for Decalso in the AOAC method. Recoveries of thiamine range from 95 to 100%, using Bio-Rex 70 (hydrogen form) ion exchange resin. The other resins tested were not satisfactory.

The determination of total thiamine in many products requires treatment of the acid extract with an enzyme preparation and subsequent purification by ion exchange chromatography (1, 2). An enzyme preparation must hydrolyze the starch present in most food samples, and release thiamine from its phosphate esters (1–3). The second function is vital because thiamine phosphates, although they are converted to thiochrome phosphates, are not extracted by isobutyl alcohol, and thus are not detected by the assay (3). Although Mylase P and Clarase T300 enzyme preparations have fulfilled this function in the past (1), they are no longer available. To find a suitable replacement, we devised a procedure for evaluating the effectiveness of an enzyme preparation in releasing thiamine from thiamine monophosphate. All enzyme preparations tested were effective in hydrolyzing the starch in the acid extracts of food samples.

After the acid extract of the product has been treated with an effective enzyme preparation, the extract frequently requires purification by ion exchange chromatography (1, 2). Decalso, which traditionally has been used for this purpose, is no longer available. After evaluating a number of ion exchange resins, we found an appropriate substitute.

METHOD

Materials

For the purpose of this report, revise **43.024** of the official method to include the following:

(**a**) *Ion exchange resins.*—

(*1*) *Bio-Rex 70* (*hydrogen form*).—Add 300 mL 2N HCl to 50 g Bio-Rex 70® (Bio-Rad Laboratories, Richmond, CA), stir 15 min, decant, and repeat. Add 300 mL water, stir 1 min, decant, and repeat until pH of water is 4.5–7.0. Water should be free of suspended resin when allowed to settle 15 s. If not, repeat water washing until clear.

(*2*) *AG® 50 W-X8.*—Prepare as in **43.024(f)** (Bio-Rad Laboratories).

(*3*) *Ionac C-102®.*—Prepare as in **43.024(f)** (MCB Chemial Co.).

(**b**) *Enzymes.*—(*1*) *Rhozyme S.*—Rohm & Haas Co. (*2*) *Acid phosphatase.*—ICN Pharmaceuticals. (*3*) *Acid phosphatase.*—Sigma Chemical Co. (*4*) *α-Amylase.*—No. 6630, Sigma Chemical Co. (*5*) *α-Amylase.*—No. 6880, Sigma Chemical Co. (*6*) *β-Amylase.*—Sigma Chemical Co. (*7*) *Pancreatin 1X.*—ICN Pharmaceuticals. (*8*) *Pepsin 1-10,000.*—ICN Pharmaceuticals. (*9*) *Clarase® T300.*—Miles Laboratories.

(**c**) *Thiamine monophosphate* (*TMP*).—Prepare as in **43.024(o)** (ICN Pharmaceuticals).

Evaluation of Enzyme Preparations

A fresh 6% solution of each enzyme was prepared in 2.5M sodium acetate. Two working solutions, one containing 1.0 μg thiamine/mL from thiamine HCl and the other containing 1.0

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μg thiamine/mL from thiamine monophosphate were prepared as in **43.024(o)** (*1–3*).

To 100 mL volumetric flask, add 10 mL thiamine HCl working solution, 50 mL 0.1N HCl, and 5 mL 6% enzyme–sodium acetate solution. To another volumetric flask, add 10 mL thiamine monophosphate working solution, 50 mL 0.1N HCl, and 5 mL 6% enzyme–sodium acetate solution. Incubate both for 2 h at 55°C, cool, and dilute to 100 mL. Filter and determine thiamine concentration by AOAC thiochrome method, **43.028–43.029.** Divide result obtained using enzymes listed by the result obtained using Clarase T300. Multiply by 100. This value represents the percent dephosphorylation compared with Clarase T300.

Evaluation of Ion Exchange Materials

Resin columns, 10 cm × 9 mm, were prepared as described in **43.024(f)**. Run an aliquot of thiamine HCl solution (at pH 4.5) containing 2.5 μg thiamine through each column as described in **43.027.** Determine thiamine concentration by thiochrome method, **43.028–43.029.** Divide result obtained when using column by result obtained without using column. Multiply by 100. This value equals the percent recovery.

Food samples were analyzed by the AOAC method, using the steps described above. In one experiment, the enzyme preparations were varied. In another, the ion exchange materials were varied.

Results and Discussion

The enzymes tested were chosen for a variety of reasons. Acid phosphatase was used by Rindi and de Giuseppe to hydrolyze thiamine phosphates (3). Acid phosphatase also is a component of Clarase T300 (4). Clarase 40,000, the replacement for Clarase T300, was tested to see if it was as effective for thiamine assay. Rhozyme S and α-amylase were tried because they are good starch saccharifying enzymes. We thought that if acid phosphatase was effective, it then would need to be diluted with a starch saccharifying enzyme. This also would lessen the cost. Other enzyme preparations available in our laboratory with some potential for replacing Clarase T300 also were tested.

Dephosphorylation of TMP by various enzyme preparations, compared with Clarase T-300 (=100%) were as follows: Clarase 40,000, 33%; α-amylase, Sigma No. 6630, 37%; β-amylase, 5.0%; pepsin, 7.0%; pancreatin, 8.5%; Rhozyme S, test 1, 95%, test 2, 97%, test 3, 91%; acid phosphatase, ICN, test 1, 11%, test 2, 16%; acid phosphatase, Sigma, lot 1, test 1, 91%, lot 1, test 2, 91%, lot 2, test 1, 5%, lot 2, test 2, 5%.

Table 1. Comparison of thiamine levels found in food products, using 3 enzyme preparations

	Thiamine, mg/100 g		
Product	Clarase T300	Rhozyme S	α-Amylase
Noodles	1.03	1.00	—
Soy protein	0.63	0.69	—
Wieners	0.21	0.24	0.14
Nuts, Type A	0.85	0.83	0.72
Nuts, Type B	0.19	0.18	0.17
Raisin bread	0.47	0.47	—
Pork strips	0.69	0.66	—
Egg product	0.28	0.30	—
Dehydrated lunch	0.17	0.16	—
Ham	0.73	0.80	—
Powdered infant formula	1.04	1.02	—

The data show that most of the enzymes tested were not effective on TMP. Rhozyme S is almost as effective as Clarase T300 but acid phosphatase varies significantly between lots and suppliers. This variation could not be explained.

The lot of acid phosphatase which gave 91% recovery of thiamine from TMP was mixed with various quantities of α-amylase to determine how far it could be diluted and still remain effective. This experiment confirmed that 20 mg of acid phosphatase added to 300 mg α-amylase was just as effective as the acid phosphatase alone.

α-Amylase appeared to be somewhat more effective in the analysis of certain foods for thiamine than it was in the release of thiamine from TMP (Table 1). Because of fluctuation of results obtained using α-amylase on foods compared with those obtained using Clarase T300, experiments with this enzyme were discontinued.

Based on the results of our experiments, Rhozyme S appeared to be the best enzyme for use in determining thiamine. The data show that Rhozyme S is effective on TMP, gives results on food samples comparable with those obtained with Clarase T300, is effective in hydrolyzing starch, and was less expensive than acid phosphatase. However, since completion of this study, we discovered that Rhozyme S is no longer available, necessitating further study.

Ion exchange resins were tested by passing aliquots of the thiamine standard solution at pH 4.5 through columns of various ion exchange materials as described in the official method. The recovery of thiamine was determined by comparing the concentration of purified standard with the concentration of the standard

Table 2. Comparison of thiamine levels found in food products, using 2 different ion exchange columns

Sample	Thiamine, mg/100 g	
	Bio-Rex 70 (hydrogen form)	Decalso
Wieners	0.19	0.19
Cheese	0.04	0.04
Ham	1.00	1.03
Noodles	0.62	0.62
Yeast	0.59	0.26
Animal feed	5.56	5.29
Beef	0.02	0.03
TV Dinner	0.02	0.02
Chinese style vegetables	0.02	0.02
Mustard	0.20	0.12
Candy bar	0.08	0.08
Bean sprouts	0.07	0.06
Egg product	0.29	0.26
Bacon	0.68	0.65
Powdered infant formula	1.04	1.04

which had not been passed through the ion exchange column. The comparative recoveries of 2.5 μg thiamine passed through 4 ion exchange resins are as follows: Decalso, 95; Ionac C-102, 89; AG 50W-X8, 25; Bio-Rex 70 (sodium form), 38; Bio-Rex 70 (hydrogen form), 98%.

The recovery of thiamine from Ionac C-102, a material similar to Decalso, was 89%. However, this material is no longer commercially available, and it was not considered further. The low recovery of thiamine from AG 50W-X8 appeared to be due to incomplete removal of thiamine from the column. An additional 60 mL eluant increased the recovery of thiamine to 85%. The need for eluant volumes of this magnitude render this resin impractical for use without further study. The low recoveries of thiamine from Bio-Rex 70 (sodium form) resulted from the instability of thiamine to the alkaline pH (10.8) which results when Bio-Rex 70 (sodium form) is suspended in water. Data indicate that the recovery of thiamine, as determined in 12 separate analyses by various analysts on different days, from Bio-Rex 70 (hydrogen form) is 95–100%, whereas the recovery of thiamine from Decalso is 90–100%.

The capacity of Bio-Rex 70 (hydrogen form) was tested by passing 5 and 25 μg thiamine through the columns. Recoveries were 100 and 99%, respectively. Fractionation tests showed the thiamine to be in the first 15 mL eluate.

Samples were analyzed for thiamine, using Decalso and Bio-Rex 70 (hydrogen form). Table 2 shows that results compare favorably. Recoveries of added thiamine from samples range from 95 to 100% for Bio-Rex 70 (hydrogen form) and for Decalso. The low results obtained using a Decalso column for yeast and mustard were due to high blanks. These low results re-occurred when these products were retested.

Recommendations

Further study of enzyme preparations is recommended.

A collaborative study is recommended using Bio-Rex 70 (hydrogen form) in place of Decalso in the official AOAC method (1).

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Comparison of Dietary Fiber Methods for Foods

MARY M. HECKMAN and SUSAN A. LANE
Ralston Purina Co., Checkerboard Square, St. Louis, MO 63188

In order to evaluate several proposed dietary fiber methods, 12 food samples, representing different food classes, were analyzed by (*1*) neutral and acid detergent fiber methods (NDF, ADF); (*2*) NDF with enzyme modification (ENDF); (*3*) a 2-fraction enzyme method for soluble, insoluble, and total dietary fiber, proposed by Furda (SDF, IDF, TDF); (*4*) a 1-fraction enzyme method for total dietary fiber proposed by Hellendoorn (TDF). Foods included cereals, fruits, vegetables, pectin, locust bean gum, and soybean polysaccharides. Results show that TDF by Furda and Hellendoorn methods agree reasonably well with literature values by the Southgate method, but ENDF is consistently lower; that ENDF and IDF (Furda method) agree reasonably well; that except for corn bran fiber (insoluble) and pectin and locust bean fiber (soluble), all materials have significant fractions of both soluble and insoluble fiber. The Furda method was used on numerous food and ingredient samples and was found to be practical and informative and to have acceptable precision (RSD values of 2.65-7.05%). It is suggested that the Furda (or similar) method be given consideration for the analysis of foods for dietary fiber.

Current interest in the nutritional aspects of dietary fiber has emphasized the need for a satisfactory analytical method; our laboratory, in common with many others, has been concerned with the problem. Ideally, the method should be simple enough to be performed routinely and should measure all or most of the constituents of dietary fiber.

Methods for measuring insoluble fiber (cellulose, insoluble hemicelluloses, and the non-polysaccharide lignin) have been greatly improved with the use, first, of detergent methods by Van Soest (1) in the 1960s, and then, in the 1970s, of enzyme-modified detergent methods by Schaller (2), Robertson and Van Soest (3), Mongeau (4), and others. However, the more soluble or hydrophilic polysaccharides are measured less frequently or not at all. Many literature references, of which only a few are cited here (5–8), mention the beneficial effects of the hydrophilic polysaccharides (soluble hemicelluloses, soluble pectins, gums) on, for example, carbohydrate and lipid metabolism. It may be that, when the physiology of dietary fiber is more completely understood, it will be possible to suggest a recommended daily allowance of dietary fiber comprised of more or less specific proportions of insoluble and soluble fractions.

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Our principal considerations in the selection of a method were, first, measurement which would include the more soluble polysaccharides, preferably differentiated from the less soluble, and second, speed and convenience of analytical method.

We of course considered the Southgate method (9), which is intended for human foods. It quantitates cellulose, lignin, and water-insoluble and water-soluble non-cellulosic polysaccharides. Defining, as it does, the fiber pattern, it provides valuable information and is considered by many workers to be the most nearly ideal method. It is, however, quite time-consuming and demands skilled technique. These factors seemed to rule it out as a practical routine method.

We then considered the enzyme methods used by a number of European workers. The first of these was reported by Williams and Olmsted in 1935 (10). More recent work has included methods by Hellendoorn et al. (11), Schweizer and Würsch (12), Asp and Johansson (13), Theander and Amen (14). These methods represent a biochemical approach, using (except for Theander) physiological enzymes which are thought to simulate animal digestion and thereby give results which correlate with the actual fate of foods in the digestive tract. They vary in details of procedure and in fractions obtained: Some methods obtain insoluble and soluble dietary fiber as separate fractions, some obtain one total amount, and some obtain insoluble dietary fiber only.

It was decided that in evaluating the methods, they would be compared with the conventional neutral and acid detergent methods (1) and with an enzyme-modified neutral detergent fiber method, which is more applicable to starchy human foods.

METHODS

Methods chosen for evaluation and comparison are given below:

(*1*) *Neutral and acid detergent fiber* (*NDF, ADF*). —These are the conventional Goering-Van Soest methods as published in U.S. Department of Agriculture Handbook No. 379 (1). They consist

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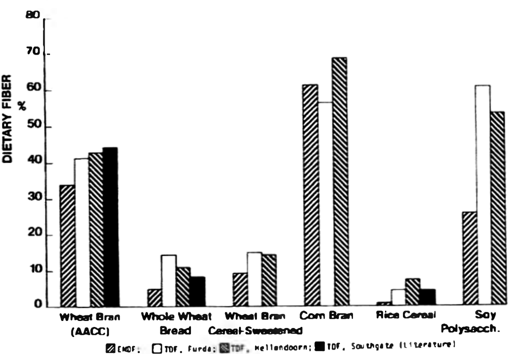

Figure 1. Total dietary fiber in cereal and legume products.

of one-stage digestions with either a neutral or an acid detergent solution. The insoluble residue from the digestion consists, theoretically, of cellulose, hemicellulose, and lignin in the NDF method, cellulose and lignin in the ADF method.

(2) *Enzyme-modified neutral detergent fiber (ENDF).*—This is the method, presented by Robertson and Van Soest at the 1977 meeting of the American Society of Animal Science (3), specifying bacterial amylase (Type III-A, from *Bacillus subtilis*, Sigma Chemical Co. No. 6505). Amylase is used to digest the starch which remains undissolved when the routine NDF digestion is applied. Note: This particular modification was chosen because of its practicality. The interfering starch is removed by enzyme action before filtration, which speeds filtration considerably.

(3) *Enzyme digestion—two fractions (Furda).*—This method was presented at the 1979 meeting of the AOAC (Abstr. No. 87) (15). Briefly, it is a digestion with 0.01N HCl, pepsin, and pancreatin (Sigma No. P7012, No. P1750; both porcine). The residue from this treatment is measured as insoluble dietary fiber (IDF); the material in the filtrate is precipitated with 4 volumes of ethanol and measured as soluble dietary fiber (SDF). Sum of IDF and SDF is total dietary fiber (TDF). It was chosen for evaluation because it seemed to meet the qualifications that had been set.

Note: Furda's method, as presented at the AOAC meeting, describes conditions for digestion with either physiological or bacterial enzymes. He shows similar results with either. Because the majority of workers specify physiological enzymes, we elected to use them.

(4) *Enzyme digestion—one fraction (Hellendoorn).*—This was published in 1975 (11). It involves digestion with 0.1N HCl, pepsin, and

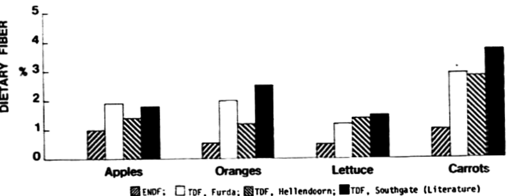

Figure 2. Total dietary fiber in fruits and vegetables.

pancreatin (Sigma No. P7012, No. P1750). The residue is measured as TDF. It was chosen for its simplicity and convenience.

(5) Literature values by the Southgate method (16, 17), where available, are included in the data.

Samples surveyed are given below.

Wheat bran	AACC Check Sample, 87.3% soft white and 12.7% club white wheat
Whole wheat bread	100% whole wheat, Earth Grains brand
Pre-sweetened wheat bran cereal	"Honey Bran," Ralston Purina Co.
Corn bran	From 1977 AACC collaborative study of Schaller enzyme modification of NDF
Rice cereal	"Rice Chex," Ralston Purina Co.
Soy polysaccharides	Soy cotyledon cell wall carbohydrates
Apples (unpeeled)	
Oranges (peeled)	
Lettuce	
Carrots	
Citrus pectin	NF Lemon Pectin No 3442, Sunkist Growers
Locust bean gum	Sigma No. G-0753

Bran and rice cereals were ground to 1 mm size before analysis. Whole wheat bread (4 slices); apples (4, cored, unpeeled); oranges (9, peeled); lettuce (iceberg, 1/4 head); carrots (6, tops and tails removed, unscraped) were freeze-dried, and then ground. Remainder of samples did not require preparation.

Results and Discussion

Results of the various measurements of dietary fiber are summarized in Table 1.

Table 1. Percent dietary fiber by several methods [a]

	Goering-Van Soest (1)		Robertson-Van Soest (3)	Furda (15)			Hellendoorn (11)	Southgate (9) lit. values
Sample	NDF	ADF	ENDF	IDF	SDF	TDF	TDF	TDF
Wheat bran (AACC)	39.2 ± 0.23	10.7 ± 0.04	33.9 ± 0.24	27.0 ± 0.36	13.1 ± 0.14	40.1 ± 0.22	42.2 ± 0.02	44.0(16)
Whole wheat bread	11.9 ± 0.28	2.1 ± 0.08	5.0 ± 0.02	10.5 ± 0.26	4.1 ± 0.14	14.6 ± 0.40	10.9 ± 0.08	8.5(16)
Pre-sweetened wheat bran cereal	11.0 ± 0.04	5.4 ± 0.09	9.4 ± 0.14	10.3 ± 0.54	4.7 ± 0.06	15.0 ± 0.61	14.9 ± 0.28	—
Corn bran	72.1 ± 0.12	15.4 ± 0.10	60.8 ± 0.16	52.8 ± 0.08	3.2 ± 0.10	56.0 ± 0.18	68.4 ± 1.08	—
Rice cereal	6.9 ± 0.08	0.5 ± 0.01	0.6 ± 0.06	1.4 ± 0.10	2.9 ± 0.14	4.3 ± 0.04	7.3 ± 0.21	4.5(16) [b]
Soy polysaccharides	30.7 ± 0.42	15.3 ± 0.03	25.3 ± 0.08	30.3 ± 0.42	29.7 ± 0.19	60.0 ± 0.62	52.6 ± 1.36	—
Apples (with peel)	1.2 ± 0.01	1.0 ± 0.03	1.1 ± 0.06	1.3 ± 0.02	0.6 ± 0.02	1.9 ± 0.01	1.4 ± 0.01	1.8(17) [c]
Oranges (peeled)	0.7 ± 0.04	0.7 ± 0.01	0.6 ± 0.04	0.9 ± 0.00	1.2 ± 0.08	2.0 ± 0.08	1.2 ± 0.04	2.5(17) [c]
Lettuce	0.7 ± 0.01	0.7 ± 0.01	0.5 ± 0.02	0.8 ± 0.00	0.4 ± 0.00	1.2 ± 0.00	1.4 ± 0.01	1.5(16)
Carrots	1.2 ± 0.02	1.2 ± 0.04	1.0 ± 0.02	1.6 ± 0.01	1.2 ± 0.01	2.9 ± 0.01	2.8 ± 0.04	3.7(16)
Citrus pectin	1.0 ± 0.10	0.6 ± 0.11	none	1.6 ± 0.05	86.6 ± 0.60	88.1 ± 0.65	(U)	—
Locust bean gum	(U)	1.0 ± 0.05	(U)	4.6 ± 0.13	77.3 ± 0.68	82.0 ± 0.54	(U)	—

[a] Original weight basis; mean of duplicate analyses.
NDF = neutral detergent fiber
ADF = acid detergent fiber
ENDF = enzyme modified NDF
IDF = insoluble dietary fiber
SDF = soluble dietary fiber
TDF = total dietary fiber
(U) = Unfilterable
[b] Rice Krispies.
[c] Pectin included.

Table 2. Distribution of insoluble and soluble dietary fiber—Furda method

Material	Insoluble, %	Soluble, %
Wheat bran (AACC)	67	33
Whole wheat bread	72	28
Pre-sweetened wheat bran cereal	69	31
Corn bran	94	6
Rice cereal	33	67
Soy polysaccharides	50.5	49.5
Apples (unpeeled)	69	31
Oranges (peeled)	43	57
Lettuce	68	32
Carrots	57	43
Citrus pectin	1.8	98.2
Locust bean gum	5.7	94.3

Table 3. Percent relative standard deviations—Furda method [a]

	Mixtures (27 samples)		Ingredients (8 samples)	
Fraction	RSD, %	Range, %	RSD, %	Range, %
Soluble dietary fiber	4.53	2–14	3.33	2.5–30
Insoluble dietary fiber	7.05	1–15	4.24	4–30
Total dietary fiber	4.69	4–29	2.65	8–60

[a] Based on duplicate samples, using one-way analysis of variance.

Inspection of the data indicates, as have many other studies, that NDF is almost invariably higher than ENDF, particularly in the high starch materials such as whole wheat bread and rice cereal. When the NDF method, which had been devised for forages, was applied to human foods, it was soon recognized that the NDF residue contained considerable amounts of starch, and the modifications referred to previously (2–4) are among many which have been proposed to correct the problem.

Figures 1 and 2 show the end results of all methods used in the survey except NDF and ADF. Examination reveals that total dietary fiber, as obtained by the Furda and Hellendoorn methods and by the Southgate method where available, are in reasonably good agreement. Enzyme-modified NDF gives lower values on all except one of the samples surveyed. It is generally accepted that the more soluble fiber components are lost with this method. Pectins are known to be solubilized by the detergent solutions used, and support of the assumption that some hemicelluloses are also solubilized is given by Baker (18).

The hydrophilic polysaccharides, pectin and locust bean gum, were measurable by the Furda method only. Pectin gave little or no residue in the detergent methods and an unfilterable residue in the Hellendoorn method; locust bean gum gave unfilterable residues by both methods. These problems could no doubt have been resolved, but no attempt was made.

Inspection of the data reveals that insoluble dietary fiber values, as measured by ENDF and the Furda method, are in reasonably good agreement. In some instances, or in some materials, the insoluble component may be the only one of interest.

Breakdown into soluble and insoluble dietary fiber was possible by the Furda method only. Table 2 shows the distribution of fractions. It will be noted that the fiber in corn bran is almost entirely in the insoluble fraction; the other materials have significant fractions of both insoluble and soluble dietary fiber.

Following is a summary of the conclusions reached as a result of the survey: (*1*) Both the Furda and Hellendoorn enzyme methods give total dietary fiber values that are in reasonably good agreement with values by the Southgate method. (*2*) Enzyme-modified NDF gives (with a single exception) lower values than either the Furda or Hellendoorn methods. (*3*) Insoluble dietary fiber values, as measured by ENDF and the Furda method, are in reasonably good agreement. (*4*) The Furda method, alone of those tested, measures both soluble and insoluble dietary fiber.

Taking all of the above into consideration, we decided to put the Furda method into use. With it, we have successfully analyzed a number of ingredients and foods, many of which contain hydrophilic polysaccharides. We have found that it has acceptable precision. Relative deviations are shown in Table 3.

We have found the method practical, as well as informative. In a research environment, we have been able to run 6 samples with 4½ h of operator attention. If insoluble dietary fiber should be all that is desired, 6 samples can be run with 3 h of operator attention. These times could undoubtedly be shortened in a control laboratory environment.

Much work remains to be done before a dietary fiber method can be considered for acceptance. In the further investigation of this method, we are analyzing more samples and will analyze the soluble and insoluble fractions for residual protein and starch as well as component monosaccharides and uronic acids. Our expe-

rience to date, however, has been encouraging and we would like to suggest that this, or a similar, method be given consideration for the analysis of foods for dietary fiber.

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STATISTICAL TECHNIQUES

Correction of Bias in Collaborative Check Sample Series Reporting

PATRICK C. KELLY
Canada Packers Inc., Research Centre, 2211 St. Clair Ave W, Toronto, Ontario, Canada M6N 1K4

Scores or accuracy indices used in collaborative check series to evaluate laboratory performance are influenced by factors that have nothing to do with performance. Scores based on normalized deviations are affected by the number of results reported on a given method. The average score or overall accuracy index obtained by a given laboratory is affected also by the number of results reported by that laboratory. By judicious choice of methods on which to report, a laboratory can improve its score without making a corresponding improvement in analytical performance. These effects are revealed in an examination of the statistical distribution of normalized deviations. The magnitude of these effects on laboratory rankings is obtained from modeling experiments with computer-generated random numbers. The inherent unfairness of the conventional method of calculating performance indices can be eliminated by converting normalized deviations to numbers having a normal statistical distribution. This transformation is mathematically complicated, but is not too difficult for a computer. The resulting parameters accurately reflect laboratory performance, are relatively easy to interpret, and permit the establishment of confidence intervals for laboratory rankings.

In a collaborative check series, homogeneous samples are sent to a number of participating laboratories for the determination of several components. Analytical results are collected and compared, and a report is issued giving a score for each analysis and an overall score and rank for each laboratory. Unlike a collaborative study, the objective of a check series is the measurement of laboratory rather than method performance. Results for many large scale check series programs are analyzed by a procedure developed by Perrin and Glocker (1), which will be referred to here as the conventional method. First, let us define the details of this procedure.

Let x_{lm} represent the result obtained by Lab. l using method m on a particular sample ($l = 1, 2, \ldots L$; $m = 1, 2 \ldots M$). Usually, some laboratories will not be able to report on all methods. Let M_l represent the number of methods reported by Lab. l, and L_m the number of results reported for Method m.

The normalized deviation for Lab. l on Method m is

$$Z_{lm} = d_{lm}/s_m \tag{1}$$

where $d_{lm} = x_{lm} - \bar{x}_m$; $\bar{x}_m$ is the mean; and s_m is the standard deviation for Method m. The mean and standard deviation usually are censored in some manner, that is, results that are obviously in error are left out of the calculations. If some results are rejected, L refers to the number of results used in calculating the mean and standard deviation.

The deviation from the mean, d_{lm}, is a measure of how closely a result agrees with the consensus value. This is divided by the standard deviation so that performances among methods having different standard deviations can be compared. The normalized deviation, in theory, places scores of all methods on an equal basis.

The square of a normalized deviation is called a score. An average of the scores for all the results reported by a laboratory, which is called the accuracy index, I_c, provides a measure of overall laboratory performance. In the conventional procedure, laboratories are ranked according to I_c. The laboratory in first place will have the smallest accuracy index. In actual practice the square root of I_c may be used for ranking, but taking the square root has no effect on the order of ranks. We will use the square form because this makes the statistics somewhat simpler.

Variations on this procedure are common. The accuracy index may be averaged over several samples to give a statistically stable indication of performance. The accuracy index may be calculated as a weighted average, the weight reflecting the relative importance of different determinations. Duplicate results may be requested for each method. From the way I_c is calculated, having duplicate results is equivalent to doubling the number of laboratories reporting on each method and doubling the number of methods reported by each laboratory. That is, L_m is twice the number of laboratories reporting

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and M_l is twice the number of methods reported.

The objective of this paper is to come to grips with the statistical properties of normalized deviations, scores, and accuracy indices so that they can be fully understood. There are 3 areas of concern. The first involves the number of results reported on each method. If the L_m values are different, are the scores really comparable between methods? Second, what is the effect of different M_l values? That is, is it fair to rank laboratories reporting on only 4 methods, say, with others reporting on 10 methods? Third, what is the role of chance in ranking? A laboratory dropping from first to fiftieth place out of a hundred laboratories, say, might consider itself in trouble. If the top 50 laboratories were in fact performing equally well, such a temporary shift could occur entirely by chance.

It will be shown that the conventional procedure can be subject to substantial error when the number of laboratories reporting on any part of a program is small. Fortunately, the large check series programs protect themselves against serious error by not evaluating methods or laboratories if the number of results reported is less than a certain minimum.

Bias in Scores

A score, Z^2_{lm}, is the square of a normalized deviation as defined by Equation *1*. The objective of this section is to examine the statistical distribution of a score for its dependence on the number of laboratories reporting, L_m. The subscripts l and m will be dropped in what follows to simplify the symbology. It will be clear from the context that the subject is either a particular laboratory, a particular method, or both.

A critical simplifying assumption will be that the data have a normal or Gaussian distribution. It is not clear what will happen when the data have a non-normal distribution. However, because most methods are subject to a number of independent sources of error, none of which will be dominant if the method has been designed carefully, the Central Limit Theorem comes into play (2). This means that the data will have a distribution not greatly different from normal. In any event, non-normal data should be converted to normal by means of a mathematical transformation. For example, taking the logarithm of log-normal data will provide data with a normal distribution.

It will be assumed for now that all laboratories perform equally well. The population of results will have a common mean μ and standard deviation σ.

The distribution of a deviation from a mean, d, will be normal and have a mean equal to zero. On first thought, one might expect that the variance of d would be σ^2. Recall, however, that d_{lm} uses datum x_{lm} in 2 ways: once as x_{lm} directly and once again in $\bar{x}_m$. Since x_{lm} and $\bar{x}_m$ are correlated, the correlation depending on L_m, the variance of d_{lm} will be less than σ^2. A little algebra will show that variance of d, which is also the mean of d^2, is $\sigma^2 (L - 1)/L$. The distribution of d^2 divided by its mean will be Chi-square with one degree of freedom. Already, we see here an insidious effect of the number of laboratories reporting.

Because the estimated variance, s^2, is made up of the sum of squares of normal random variables, the distribution of $(L - 1)s^2$ will be Chi-square with $L - 1$ degrees of freedom.

A score, Z^2, being the ratio of 2 Chi-square variables, should have a Fisher's F distribution with 1 and $L - 1$ degrees of freedom. This is not so, however, because d^2 and s^2 are not independent. Recall that s^2 is the sum of squared deviations, one of which is d^2 itself. A score, Z^2_1, say, may be written as follows.

$$\begin{aligned} Z^2_1 &= (L - 1)d^2_1/(d^2_1 + d^2_2 + d^2_3 \ldots) \\ &= (L - 1)/[1 + (d^2_2 + d^2_3 \ldots)/d^2_1] \qquad (2) \end{aligned}$$

The numerator of the ratio of d^2 values in Equation 2 is related to a standard deviation calculated from the sum of squared deviations leaving out d^2_1. This observation provides a clue to handling the statistics of a score.

Define a new score as follows:

$$F_j = \frac{(L - 1)d'^2_j}{Ls'^2_j} \qquad (3)$$

where $d'_j = x_j - \bar{x}'_j$; and $\bar{x}'_j$ and s'_j are calculated from the data excluding x_j. Clearly, d'^2_j and s'^2_j are independent. Their ratio, therefore, times a factor involving L that accounts for the standard deviation of d'_j not being equal to σ, has a Fisher's F distribution with 1 and $L - 2$ degrees of freedom.

After a considerable amount of arithmetic, it can be shown that

$$F_j = \frac{(L - 2)d^2_j}{as^2 - d^2_j}$$

or, on dropping the subscript and rearranging

$$F = (L - 2)h/(1 - h) \qquad (4)$$

where $h = Z^2/a$, and $a = (L - 1)^2/L$. Equation 4

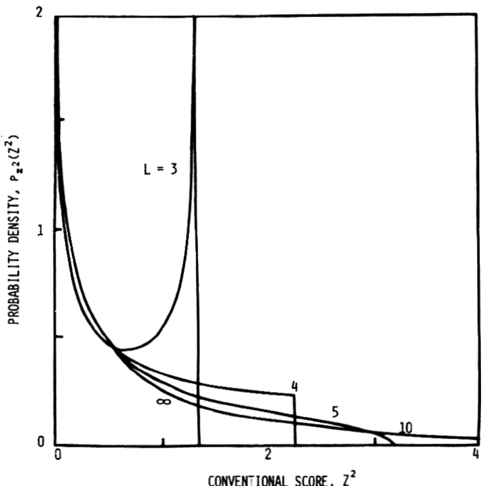

Figure 1. Distribution of conventional scores.

can be solved for Z^2, giving

$$Z^2 = aF/(F + L - 2) \qquad (5)$$

David et al. (3) give a similar formula for the ratio of a range to a standard deviation.

It would seem that by dropping one of the data points in the calculation of F via Equation 3, we are throwing data away. This is not so. Recall that d and s are not independent. The transformation defined by Equation 4 gives a one-to-one correspondence between Z^2 and F; no information is lost. The transformation is merely a change from an as yet undefined distribution to a more familiar ratio of variances—Fisher's F distribution.

The probability-density function of F is

$$p_F(F) = \frac{\nu^{\nu/2}F^{-1/2}}{B(1/2, \nu/2)(F + \nu)^{(\nu+1)/2}} \qquad (6)$$

where B is the beta function with parameters 1/2 and $\nu/2$, and ν is the degrees of freedom, $L - 2$ (2).

The distribution of a score, Z^2, can be obtained from Equations 5 and 6 and the formula for the transformation of variables (2).

$$p_{Z^2}(Z^2) = p_F(F(Z^2))dF/dZ^2 \qquad (7)$$

$$= \frac{h^{-1/2}(1 - h)^{(L-4)/2}}{aB(1/2, (L - 2)/2)} \qquad (8)$$

where $h = Z^2/a$; $a = (L - 1)^2/L$; and L is the number of results reported for a particular method after censoring. This is a beta distribution of the first kind. It is not often encountered in analytical chemistry, but it will arise in the estimation of success rates in data having a binomial distribution (cure rate of drugs, kill rate of insecticides, etc.).

The distribution of Z^2 is illustrated in Figure 1. Many of its properties are rather remarkable and not at all in accordance with intuition. An unexpected property is that Z^2 has an abrupt upper limit, $(L - 1)^2/L$. The limit is a mathematical property of Z^2 that is not influenced by the assumption of normality in the raw data. The limit is reached when all but one of the results for a method are equal.

The distribution has a pole at the origin for all values of L. The shape of the distribution changes abruptly as L goes from 3 to 5. Above $L = 5$ the changes are more gradual. For values of L above about 20, the distribution is approximately Chi-square.

The mean of Z^2 is $(L - 1)/L$. Note that the mean increases with L. The standard deviation of Z^2 is $[2a(L - 2)/(L(L + 1))]^{1/2}$, which also increases with L. For $L = 3$, 10, and 20, the standard deviations are 0.47, 1.09, and 1.24, respectively.

These properties show that, on the average, lowest and hence best scores are obtained on methods having the least number of reported results. A canny laboratory manager can ensure a favorable score by choosing to report only the least popular methods. This will be unfair to laboratories not adopting the same strategy. Clearly, Z^2 is a biased indicator of laboratory performance.

Bias in Normalized Deviations

Since $|Z| = \sqrt{Z^2}$, the distribution of a normalized deviation can be obtained from Equation 8 and the formula for transformation of variables,

$$p_Z(Z) = \frac{(1 - h)^{(L-4)/2}}{\sqrt{a}\, B(1/2,(L-2)/2)} \qquad (9)$$

where h, a, and B are defined previously. The right half of the distribution is illustrated in Figure 2. It is a symmetrical bell-shaped curve that slowly approaches the normal form as L increases. It has limits $\pm\sqrt{a}$, a mean equal to zero, and a standard deviation equal to $[(L - 1)/L]^{1/2}$.

Unfairness in the use of normalized deviations can arise in procedures for rejection of improbable data. One such procedure is to reject if $|Z| > 3$. If Z had a normal distribution, this would correspond to rejecting data outside a confidence interval having a 99.7% probability. However, Z is not normal. The fact that it has bounds

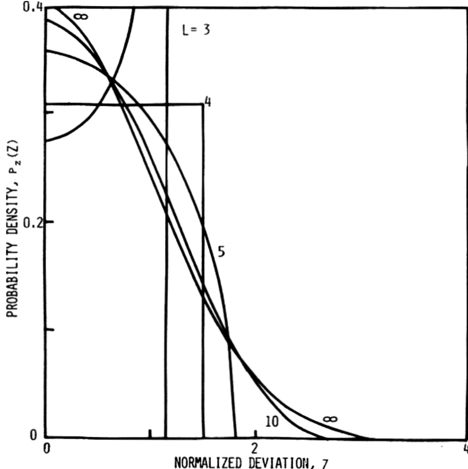

Figure 2. Distribution of normalized deviation.

means that values of Z can never exceed 3 when less than 11 results are reported. For $L = 20, 50$, and 120, the actual confidence probabilities for $|Z| > 3$ are 99.95, 99.83, and 99.77%, respectively.

The common practice of reporting averages of Z over a pair of results on the same sample or over several samples is done to provide a stable indication of analytical bias. The distribution of such a score is extremely complicated, and is especially so when the Z values have different L values. Nevertheless, a few generalizations can be made. Its mean will be zero, and its limits will be the average of the limits of the Z values. Its variance will be the sum of the variances of each Z divided by the number of Z values averaged. If the L values are different, it will be difficult to give a statistical interpretation to such an average. This is unfortunate because analysts will want to make statistical statements about the presence of analytical bias in their results.

Bias in Laboratory Rankings

Laboratories are ranked in the order of their average score, I_c, which we will call the conventional index. The average score is the sum of Z^2 values for all methods reported by a given laboratory divided by the number of methods reported by that laboratory, M. The conventional index is made up of the sum of M random variables each of which has a beta distribution dependent on L, the number of results reported for that method.

If all laboratories report on the same methods, the distribution of scores and hence the distribution of I_c will be the same for each laboratory. All laboratories will be treated equally and no bias will be introduced by the scoring system. This is not likely in practice, though. Some laboratories will not be able to report on all methods. The M and L values will be different. The scores will have different means and standard deviations. Methods having different L values, therefore, will not be given equal statistical weight in calculating I_c. A bias will be introduced into I_c that does not reflect true laboratory performance.

The bias introduced by different M and L values can be obtained from the distribution of I_c. Being a function of M parameters, however, the distribution will be exceedingly complex. Because I_c is based on the simple sum of random variables, a few generalizations can be made. The upper limit of I_c will be the average of the upper limits of the Z^2 values used in its calculation. The mean of the distribution will be the average of the mean of the Z^2 values. Its variance will be the sum of the variances of the Z^2 values divided by M. If M is greater than one, the distribution will not have a pole and will approach zero at the limits of its range. It will be a bell-shaped curve with a degree of skew to the right. The properties of most concern are that its mean will increase as the L values increase and that its standard deviation will decrease as M increases.

If the distribution of I_c could be worked out exactly, it would be possible to determine confidence intervals and thereby make statistical statements about the performance of a given laboratory. Without some kind of statistical statement, it is difficult to interpret a given value of I_c. If I_c is used as a ranking index, however, the resulting rank is easy to interpret. A laboratory in first place is obviously better than the laboratory in last place. (The error of this kind of thinking is discussed in a following section.)

The problem now is to determine the effect of L and M on rankings. In theory, the statistical distribution of a rank could be worked out but, as for I_c, this is much too complicated to formulate exactly. It is possible, though, to make generalizations from the properties of the distribution of I_c. Because the mean of Z^2 and hence the mean of I_c decreases as the L values decrease, low L values will favor a low rank. Because the skew to the right and the standard deviation of I_c increase as M decreases, a low value of M favors a low rank. In other words, to obtain a lower or better rank one should report

only on the least popular methods and report as few methods as possible. Clearly, ranks based on I_c do not entirely reflect laboratory performance.

Experimental.—To obtain estimates of the bias in ranking caused by the use of I_c, experiments were run using pseudo-random data generated by a Hewlett-Packard Model 9825 calculator. The calculator's built-in random number generator will produce independent random numbers having a uniform distribution ranging between zero and one, if the seed or initial value is chosen appropriately. To minimize the effect of choosing a bad seed, which would result in correlated numbers, a different seed was chosen for each experiment.

Random data having a normal distribution with zero mean and unit standard deviation were calculated by means of the polar method described by Box and Muller (4). Let $c = \sqrt{-2\ln a}$ and $f = \sin(2\pi b)$ where a and b are a pair of computer-generated uniform random numbers. A pair of simulated results were calculated from the formula: $x_1 = cf, x_2 = c\sqrt{1 - f^2}$.

An experiment consisted of obtaining a set of random numbers, assigning these to imaginary methods and laboratories, and then calculating means, standard deviations, scores, and ranks as described in the previous sections. The experiments were repeated a large number of times to obtain stable estimates of average scores and ranks. An experiment involving 10 laboratories and 10 methods repeated 5 thousand times would require about 12 h of computation time.

For L laboratories and K repetitions, an average rank, assuming no bias, will have an approximately normal distribution with population mean $(L + 1)/2$ and standard error $[(L^2 - 1)/(12K)]^{1/2}$. This can be derived from formulas given by Youden (5). In experiments to detect bias, K was chosen large enough so that the deviation of a given rank from the expected rank was at least 4 standard errors.

Results and Discussion.—Consider first the effect of L when M is the same for all laboratories. This can be done by filling out the matrix of data in blocks; represented in practice by a block of laboratories reporting on one set of methods (moisture at 125°C and fat by indirect extraction, for example) and the remainder reporting on another set (moisture by vacuum oven and fat by direct extraction). Suppose 20 laboratories report on a total of 20 methods. Labs 1, 2, and 3 (Group A) report on Methods 1 to 10 only, and the remaining laboratories report on Methods 11 to 20 only. For both groups, $M = 10$; for each method reported by Group A, $L = 3$; and for Group B, $L = 17$. The experimental means and standard deviations of I_c averaged 0.67 (0.67 expected) and 0.15 (0.15) for each laboratory in Group A and 0.94 (0.94) and 0.38 (0.38) for Group B. The agreement between experimental and theoretical values was excellent. The average rank was 6.5 for the laboratories in Group A and 11.2 for Group B. Compare these to an average of 10.5 if I_c were a fair index for ranking. The bias in ranking, ΔR, for Group A was −4.0 ranks or −20% relative to all 20 laboratories. The error in this case was rather severe. If Group A increases to 5 members and Group B decreases to 15 members, increasing the similarity between groups, ΔR decreases to −1.5 ranks or −7.5% for Group A. When both groups have the same number of laboratories, the bias becomes indistinguishable from zero as expected.

A patently artificial pattern of data will have to be used to demonstrate the effect of M when L is the same for all methods. This can be done by filling in the data in a diagonal pattern. Lab. 1 reports on Method 1 only, Lab. 2 on Methods 1 and 2, and so on up to Lab. 6. Lab. 7 reports on all but Method 1, Lab. 8 on all but Methods 1 and 2, and so on up to Lab. 11, which reports on Method 6 only. The mean of I_c is 0.83 for each laboratory since $L = 6$ for each method. The standard deviation varied from 0.89 for Labs 1 and 11 ($M = 1$) to 0.36 for Lab. 6 ($M = 6$). The experimental and theoretical results were again identical to 2 decimal places. Since the mean of I_c is the same for each laboratory, one would expect that the average rank would be the same for each laboratory. Bear in mind, however, that the distribution of I_c is skewed to the right and that the skew is greater for lower values of L. The distribution of I_c for Labs 1 and 11 will be similar to that for Z^2 shown in Figure 1. These laboratories are likely to be ranked either first or last in each trial, but more often first because very low values of I_c are highly probable. The distribution of I_c for Lab. 6, on the other hand, will be peaked near the mean and extreme values will be unlikely. Lab. 6 should be ranked near the middle most of the time. The experimental results confirmed these predictions. Labs 1 and 11 received ΔR of −0.7. As M increased, the bias increased gradually to +0.4 for Lab. 6. Evidently, one can obtain a favorable rank by reporting as few results as possible.

This observation breaks down, however, when L is not the same for all methods. For example, when 10 laboratories reported on 5 methods and 3 of these laboratories reported on 5 additional

methods, the ΔR for these 3 laboratories was -0.3. Recall that as L increases, both the mean and standard deviation of I_c increase; whereas as M increases, the mean is not directly affected and the standard deviation decreases. The best overall strategy is to choose methods having a low average score (low L) and report as many of these as possible so that the distribution of I_c will be concentrated around its mean.

There can be no doubt that ranks based on the conventional accuracy index do not reflect laboratory performance alone when the number of results reported differ from method to method and from laboratory to laboratory. When M and L are large and the differences small, bias introduced by the use of I_c will be small. Nevertheless, bias, no matter how small, is an anathema to analytical chemists, especially when it is correctable.

Correction of Bias

The correction procedure is based on transformations of variables so that the new variables will be independent of L or M. The transformation of raw data into normalized deviations is a similar type of transformation in that dividing a deviation from a mean by a standard deviation makes the resulting variable independent of differences in precision between methods.

Scores.—Using F defined by Equation 4, the probability of obtaining a score between zero and F may be obtained as follows.

$$P_F(F) = \int_0^F P_F(v)dv \qquad (10)$$

where $P_F(v)$ is the probability density function for Fisher's F distribution with $\nu_1 = 1$ and $\nu_2 = L - 2$ degrees of freedom (Equation 6). A similar probability for a normal random variable is defined by Equation *11*.

$$P_g(g) = \int_{-\infty}^{g} p_g(u)du \qquad (11)$$

where $p_g(g)$ is the probability density function of a normal random variable, g.

$$p_g(g) = (1/\sqrt{2\pi})e^{-g^2/2} \qquad (12)$$

having, for the sake of convenience, zero mean and unit standard deviation.

If $P_g(g)$ is set equal to $P_F(F)$ and Equation *11* is solved for g, the resulting value will be a normal random variable corresponding to F. That is, having obtained F from the data, we calculate the probability of obtaining a value as high as F, and then calculate the value of a normal variable having this same probability. For any pair of values of F and L, there will be one and only one value of g. Because g is independent of L, g is precisely the variable we need as an unbiased indicator of laboratory performance.

By this roundabout way, an exact transformation of F to g may be effected. Several approximate direct transformations were tried (Equations 26.6.14 and 26.6.15 in reference 6 and the Peizer-Pratt approximation in reference 7) but without success. Bias in ranking using these approximations was often worse than that of the conventional index. Having $\nu_1 = 1$ is a severe test of approximations that are designed to be accurate for large degrees of freedom. Fortunately, the exact formulas for $P_F(F)$ are not difficult for a modern computer and reasonably accurate approximations to the solution of Equation *11* are available.

Exact formulas for $P_F(F)$ may be obtained from Equations 26.6.2, 26.6.4, and 26.6.8 by Abramowitz and Stegun (6). Note that L must be greater than 2. For even L,

$$x = 1/(1 + (L-2)/F)$$

$$B_1 = 1$$

$$B_j = 1 + (1-x)B_{j-1}(L-2j-1)/(L-2_j)$$

where $j = 2, 3 \ldots, K = (L-2)/2$

$$P_F(F) = \sqrt{x}B_K$$

For odd L,

$$y = F/(L-2), x = 1/(1+y), \theta = \arctan\sqrt{y}$$

$$B_1 = 0$$

$$B_j = 1 + xB_{j-1}(L-2j+1)/(L-2_j+2)$$

where $j = 2, 3 \ldots K = (L-1)/2$

$$P_F(F) = (\theta + \sqrt{y} \times B_K)2/\pi \qquad (13)$$

The normal variate corresponding to a probability $P_g(g)$ is given approximately by Equation 26.2.23 in Abramowitz and Stegun (6).

If $P_g(g) < 0.5$, let $p = P_g(g)$, otherwise, let $p = 1 - P_g(g)$.

$$A = c_0 + r(c_1 + rc_2), r = \sqrt{\ln(1/p^2)}$$

$$B = 1 + r(d_1 + r(d_2 + rd_3))$$

$c_0 = 2.515517$ $d_1 = 1.432788$

$c_1 = 0.802853$ $d_2 = 0.189269$

$c_2 = 0.010328$ $d_3 = 0.001308$

$$g = -r + A/B, \text{ if } P_g(g) < 0.5$$

$$g = r - A/B, \text{ otherwise} \qquad (14)$$

The error in this approximation is less than 4.5 × 10^{-4}, which is more than adequate for the analysis of check series.

The complete procedure may be represented by the sequence of transformations:

$$x \underset{1}{\rightarrow} Z \rightarrow Z^2 \underset{4}{\rightarrow} F \underset{13}{\rightarrow} P_F(F) = P_g(g) \underset{14}{\rightarrow} g,$$

where the arrows represent transformations and the numbers designate equations. For reasons involving sensitivity to bias that will be described later, it turns out that this sequence need not be carried out in its entirety.

Normalized Deviation.—The square root of F (with appropriate sign) will have the familiar Student's t distribution. Such a t value, though, will still depend on L and will require statistical tables for its interpretation. A transformation of t to a normal variate, Z', carried out in the same way that F was transformed, will eliminate these difficulties.

An equation for the probability distribution of t, $P_t(t)$, may be derived directly but it is simpler to use the expression $P_t(t) = (P_F(F) + 1)/2$. If $P_t(t)$ is equated to $P_g(g)$ in Equation *14* and the value of g so obtained given the sign of Z, the resulting variable, Z', will correspond to a normalized deviation but will have a normal distribution with zero mean and unit standard deviation.

The "normalized" normalized deviation, Z', is much more easily interpreted than Z. Unlike Z, it has no limits and it is independent of L. As shown in Figure 3 for positive Z, the transformation makes small values of Z slightly smaller and values larger than about 1 much larger. The transformation is a sequence of transformations, which may be represented as follows:

$$x \underset{1}{\rightarrow} Z \rightarrow Z^2 \underset{4}{\rightarrow} F \underset{13}{\rightarrow} P_F(F) \rightarrow P_t(t)$$

$$= P_g(g) \underset{14}{\rightarrow} g \rightarrow Z'.$$

Accuracy Index and Rank.—With normal variables to work with now, the calculation of an accuracy index independent of M is straightforward. Define a new variable, X, as follows:

$$X = \sum_{m=1}^{M} Z'^2 \qquad (15)$$

This sum of squares of normal variables will have a Chi-square distribution with M degrees of freedom. The X value still depends on M but it can be transformed to a normal variable as was done with F and t.

The probability of X needed for the transformation is taken from formulas given in Abramowitz and Stegun (6, Equations 26.4.4 and 26.4.5).

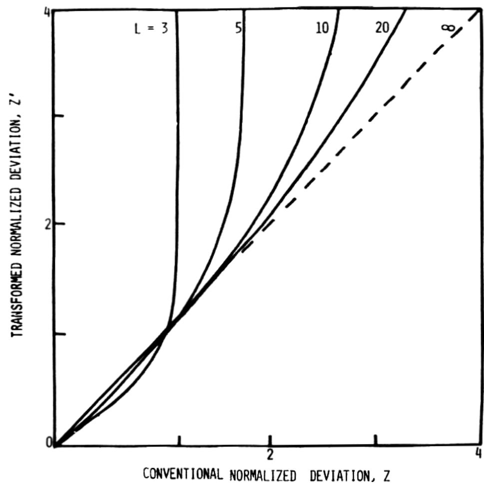

Figure 3. Normalization of normalized deviation.

For even M,

$$B_0 = 1$$

$$B_j = 1 - XB_{j-1}/(M - 2j)$$

where $j = 1, 2 \ldots K = (M - 2)/2$

$$P_X(X) = 1 - B_K e^{-X/2}$$

For odd M, the normal probability integral must be evaluated for X. This can be obtained from an approximation in Abramowitz and Stegun (6) (Equation 26.2.17).

Let $x = 1/(1 + y\sqrt{X})$, $y = 0.2316419$

$$A = x(b_1 + x(b_2 + x(b_3 + x(b_4 + xb_5))))$$

$b_1 = 0.319381530$ $\qquad b_4 = 1.821255978$

$b_2 = 0.356563782$ $\qquad b_5 = 1.330274429$

$b_3 = 1.71477937$

$$P_g(\sqrt{X}) = 1 - (A/\sqrt{2\pi})e^{-X/2}$$

The error in this approximation is less than 7.5 × 10^{-8}.

Then, for the evaluation of $P_X(X)$ for odd M,

$$B_0 = 0$$

$$B_j = 1 + XB_{j-1}/(M - 2j + 2)$$

where $j = 1, 2 \ldots K = (M - 1)/2$

$$P = (2/\sqrt{2\pi})\sqrt{X}\, B_K e^{-X/2}$$

$$P_X(X) = 2P_g(\sqrt{X}) - P - 1 \qquad (16)$$

This is then set equal to $P_g(I_p)$ and solved for I_p via Equation *14*. The resulting value, I_p, called the performance index, corresponds to the conventional index, I_c, but has a normal distribution with zero mean and unit standard deviation when the performances of all laboratories are the same on the average. The sequence of operations required to calculate I_c may seem complicated, but, when considered one step at a time, the procedure is more comprehensible:

$$x \underset{1}{\rightarrow} Z \rightarrow Z^2 \underset{4}{\rightarrow} F \underset{13}{\rightarrow} P_F(F) \rightarrow P_t(t)$$

$$= P_g(g) \underset{14}{\rightarrow} g \rightarrow Z' \underset{15}{\rightarrow} X \underset{16}{\rightarrow} P_X(X)$$

$$= P_g(I_p) \underset{14}{\rightarrow} I_p.$$

The performance index is independent of M and the L values for each method when all laboratories have the same performance. When performances are not the same, that is, when the population mean or standard deviation for results on any method by any laboratory are different from those of the other laboratories, values of I_p less than one denote better than average performance. There will be a dependence on L when one or more laboratories have an analytical bias. More will be said about this in the next section.

There was no detectable bias in ranking according to I_p in the modeling experiments reported above and in many other experiments that are not presented here. For example, in the experiment where $L = 6$ for each laboratory and M varied from 1 for Lab. 1 to 6 for Lab. 6, the errors in rank for Labs 1 and 6 were +0.032 and −0.029 ranks (10 000 trials). Compare this with errors of −0.7 and +0.4 when the conventional index was used for ranking.

If the number of trials in an experiment becomes large enough, statistically significant bias probably would be detected due to the numerical approximations used in Equations *14* and *16*. The magnitude of the bias, however, has no practical significance. The performance index, therefore, reflects laboratory performance only and is not sensitive to the number of laboratories or to the number of methods reported in a check series.

If results are to be weighted in Equation *15* according to the importance of a method, the above procedure cannot be used because the resulting X value will not be Chi-square. A sum of weighted g values (Equation *14*) divided by the square root of the sum of the weights can be used as an accuracy index.

Duplicate Data.—In the conventional system, duplicate data are treated in a way that is equivalent to L being twice the number of laboratories reporting and M being twice the number of methods reported. That is, for a pair of results x_i, and x_j reported by a particular laboratory for a particular method, 2 normalized deviations are calculated, Z_i and Z_j. In calculating I_c, the squares of these 2 values are treated exactly as if they were singlet data from 2 methods.

In the procedure proposed here, 2 values of F could be calculated, F_i and F_j. These cannot be treated as being from 2 independent methods, however. The standard deviation, s is common to both; F_i is a function of x_j and F_j of x_i. They can be made statistically independent by dropping both x_i and x_j in the calculation of the mean and standard deviation. We define a different F for duplicate data as follows:

$$F_i' = \frac{(L-2)d_i''^2}{(L-1)s_k''^2} \quad (17)$$

where $d_i'' = x_i - \bar{x}_k''$, and $\bar{x}_k''$ and s_k'' are the mean and standard deviation obtained when both components of a pair x_i and x_j are excluded. A similar equation may be written for F_j'. Note that $\bar{x}_k''$ and s_k'' are the same for both F_i and F_j'. Equation *18* may be used for calculation purposes.

$$F_i' = \{(L-3)[(L-1)Z_i + Z_j]^2\}/ \\ \{(L-2)[(L-1)(L-2) \\ - (L-1)(Z_i^2 + Z_j^2) - 2Z_iZ_j]\} \quad (18)$$

In view of the complexity of Equation *18*, we will forego the development of the joint probability distribution of Z_i^2 and Z_j^2 needed to evaluate their effect on the conventional index, I_c.

Equation *18* provides a variable with a Fisher's F distribution with one and $L - 3$ degrees of freedom. We can proceed in the now familiar manner to convert F' to a probability and then to a "normalized" normalized deviation, Z'. Equation *13* may be used for this purpose if $L - 1$ is substituted for L. The pair of values Z_i' and Z_j' may be used in the calculation of the performance index, I_p, as if they were obtained from 2 different methods.

Effect of Analytical Bias

So far it has been assumed that all laboratories perform equally well. Suppose now that one laboratory has an analytical bias. If Lab. 1 has this distinction, then $\bar{x}_1 = \mu + \sigma b$ where b is the bias. In the presence of bias, the distribution of Z will not have the form of Equation *9*. Consequently, Z' will not have a normal distribution.

Table 1. Effect of analytical bias on normalized deviation

Bias	L	$\bar{Z}_t$[a]	Z		Z'	
			Av.	SD	Av.	SD
0	3	0	0	0.82	0	1
1	3	0.67	0.59	0.64	0.67	0.84
2	3	1.33	0.93	0.34	1.17	0.67
0	5	0	0	0.89	0	1
1	5	0.80	0.77	0.67	0.85	0.82
2	5	1.60	1.24	0.43	1.50	0.69
0	10	0	0	0.95	0	1
1	10	0.90	0.84	0.86	0.90	0.95
2	10	1.80	1.51	0.66	1.69	0.85
0	20	0	0	0.97	0	1
1	20	0.95	0.93	0.90	0.96	0.96
2	20	1.90	1.72	0.79	1.84	0.91

[a] $\bar{Z}_t = \overline{(x - \bar{x})} / \sigma$.

It will be difficult to predict the effect of bias on either Z or Z'.

The results of modeling experiments for Lab. 1 with bias in the data of Lab. 1 only are given in Table 1. Since the average of results for a given method, $\bar{x}$, will be influenced by the biased result, the average deviation from the mean divided by σ will not be b, but $b(L - 1)/L$. This is given in Column 3 of Table 1 as $\bar{Z}_t$ for comparison purposes. The averages of Z and Z' increase as bias increases, but Z' is closer to $\bar{Z}_t$. The standard deviations decrease with increasing bias. This is a consequence of the existence of limits for Z. As bias increases, normalized deviations are crowded more and more toward the upper limit. The rate of decrease of standard deviation is greatest for $L = 3$, since the effect of the upper limit on the distribution of Z is greatest for low L. The standard deviations of Z' are not affected by bias as much as those of Z.

Table 2 presents the results of modeling experiments for bias in Z^2. These are compared to $I_p(M = 1)$ rather than Z'^2, since it is I_p that is used for ranking purposes. As one would expect and desire, averages of Z^2 and I_p increase as the magnitude of bias increases. For the larger values of L, the increase is greater for Z^2 than for I_p. Since we would want to downgrade biased results as much as possible, it appears that Z^2 would be a better index for ranking than the performance index, I_p. Averages, though, do not tell the whole story. Standard deviations play a part also. Unlike the standard deviations of Z, which steadily decreased as bias increased for all values of L, those for Z^2 decrease for $L = 3$ but increase with increasing bias for larger values of L. The standard deviations for I_p, on the other hand, are relatively constant with changes in bias and L.

In Table 2, Q is a measure of sensitivity to bias, combining the effects of both average and standard deviation. According to this measure, I_p is slightly less sensitive to bias than Z^2 for $L = 3$, slightly more sensitive for $L = 5$, and definitely more sensitive for larger L.

Table 2. Effect of analytical bias on score

Bias	L	Z^2			I_p		
		Av.	SD	Q[a]	Av.	SD	Q
0	3	0.67	0.47	0	0	1.00	0
1	3	0.75	0.46	0.18	0.16	0.97	0.17
2	3	0.97	0.39	0.77	0.61	0.85	0.72
0	5	0.80	0.80	0	0	1.00	0
1	5	1.09	0.90	0.32	0.33	1.05	0.32
2	5	1.70	0.90	1.00	0.99	0.95	1.04
0	10	0.90	1.08	0	0	1.00	0
1	10	1.46	1.45	0.38	0.43	1.10	0.39
2	10	2.76	1.73	1.08	1.31	1.03	1.27
0	20	0.95	1.24	0	0	1.00	0
1	20	1.73	1.87	0.42	0.50	1.13	0.44
2	20	3.62	2.58	1.03	1.46	1.10	1.33

[a] $Q(y) = (\bar{y}_{bias} - \bar{y}_0) / s_{bias}$.

The sensitivity factor, Q, should reflect the performances of I_c and I_p as ranking indices and this was found to be so. With $b = 2$ in the results of Lab. 1 for Method 1 only, and when 3 laboratories report on 5 methods, I_c gave an average rank 2.19 for Lab. 1 and I_p gave a rank of 2.18. In this case, I_c was slightly more sensitive to bias than I_p. When the number of laboratories was increased to 10, the average ranks for Lab. 1 were 7.31 using I_c and 7.34 with I_p. For 20 laboratories, these were 15.07 and 15.15. For large L, I_c is less sensitive to bias than I_p. The differences are too small, though, to have a noticeable effect in practice.

Evidently, then, the conventional index and the performance index are almost equally sensitive to bias.

An average of the normalized form of F obtained by use of Equations *13* and *14* can be used for ranking purposes. Although this index gave no evidence of bias in ranking when L values and M varied among methods and laboratories, it was slightly less sensitive to bias than I_c.

Practical Consequences of the Use of the Normalization Scheme

Detection of Outliers.—The use of a simple statistical test, such as $|Z'| > 3$, to detect erroneous results can be applied uniformly to all results. The confidence interval for such a test will have a probability that does not depend on the number of results reported. This cannot be said of the use of the normalized deviation, Z.

The Z' value for a rejected result, x_r, may be calculated as follows.

$$F_r = \frac{Ld_r^2}{(L+1)s^2} = \frac{L}{(L+1)} Z_r^2 \qquad (19)$$

Apart from a factor involving L, a conventional score for a rejected result is automatically an F value. The calculation of Z'_r may then proceed as for a non-rejected result except that L in Equation *13* should be replaced by $(L + 1)$.

Moving Averages.—Averages of Z' over several samples are more easily interpreted than similar averages of normalized deviations. An average over S results, $\overline{Z}'_s$, will have a normal distribution with standard deviation $1/\sqrt{S}$.

There may be some merit in reporting $\overline{\overline{Z}}' = \overline{Z}'_s\sqrt{S}$, that is, in standardizing an average to unit standard deviation. In the presence of bias, $\overline{\overline{Z}}'$ will increase with S. Significant analytical bias will then be easier to detect. For example, suppose that Z' was 2 in each of 3 successive samples. We get $\overline{Z}'_s = 2$ for $S = 1, 2,$ and 3. It is not obvious that the average over 3 samples is significantly different from zero. For $\overline{\overline{Z}}'$, on the other hand, the corresponding values are 2.0, 2.8, and 3.5. Because the standard deviation is 1 for each of these values, it is readily apparent that the average over 3 samples shows significant analytical bias.

An arithmetic average of I_p could be used as an index of performance over several samples. If the number of methods reported by a given laboratory is different for each sample, a simple arithmetic average will not give the correct weight to each sample. Moreover, the standard deviation of such an average will change with the number of samples averaged and, as with the average of Z', analytical bias will not be readily apparent. A better procedure would be to use the sum of $\overline{\overline{Z}}'^2$ for each method over each sample. The value of M to be used in Equation *16* for the calculation of this cumulative probability index is the sum of the M values for each sample. In other words, the cumulative performance index is obtained by treating the samples as if they were one sample with additional methods.

The cumulative performance index calculated in this way will continue to have unit standard deviation, and, as with $\overline{\overline{Z}}'$, the effects of analytical bias will accumulate. Another advantage of this system is that the number of samples accumulated need not be the same for each laboratory. Beginning laboratories can be ranked alongside veteran laboratories that have reported on 3 or more samples.

In reporting cumulative performance indices, it would be advisable to report the sum of M values as well so that the power of a test for bias could be calculated if desired. The power of a test is a statistical term for the probability that a laboratory is biased when in fact it is not. Although beyond the scope of this work, the power of a test is discussed in many statistical texts. One such is Kendall and Stuart (8).

Confidence Intervals for Ranks.—It is meaningless to make any but statistical statements about the results of a check series. We cannot state that the performance of a given laboratory is better than those of the other laboratories. We can state, however, that a given laboratory has or has not a performance index that is significantly better than those of the others at some probability level, 95%, say. A performance index and M will provide all the information needed to make such statistical statements.

Unfortunately, analysts have to report to superiors who may not understand the niceties of statistical expression. Ranks are more easily

Table 3. Conventional and performance indices compared in an actual check series

		Conventional system		Proposed system		
Lab.	M[a]	I_c	Rank	I_p	Rank	Probable rank
1	6	1.20	10	0.97	10	4–10
2	6	0.64	2	−1.21	2	1–9
3	7	0.99	8	0.25	8	1–10
4[b]	6	1.25	—	1.25	—	—
5	7	0.93	7	0.03	6	1–10
6	6	0.63	1	−1.18	3	1–9
7	6	0.76	5	−0.68	5	1–10
8	5	1.02	9	0.42	9	1–10
9[b]	6	1.39	—	1.64	—	—
10	7	0.64	3	−1.28	1	1–9
11	6	0.69	4	−0.92	4	1–10
12	5	0.93	6	0.07	7	1–10

[a] L = 12, 12, 12, 11, 10, 9, and 7 for 7 methods.
[b] Laboratories with rejected result not included in calculation of indices.

understood than performance indices. But it is too easy to come to the conclusion that the laboratory in first place, say, is better than the other laboratories. Its performance index may in fact be better than those of all, 10, or even none of the other laboratories. Ranks by themselves cannot be used to answer meaningful statistical questions.

It would be desirable to report along with a rank some kind of parameter that expresses the effect of chance. Since I_p has a standard normal distribution, a confidence interval for ranks can be established relatively easily. It was shown above that the standard deviation of I_p is slightly dependent on the magnitude of analytical bias. This effect is small enough to be ignored. The 95% confidence interval for a performance index then will be $I_p \pm 1.96$. Laboratories having a performance index outside this range perform significantly better or poorer than the given laboratory. The rank of laboratories lying next to and inside this interval may be said to form the 95% (at most) confidence interval for the rank of the given laboratory. For the sake of brevity, we will call this interval the probable rank.

If all the laboratories in a series perform equally well, the probable rank for each laboratory will include all of the laboratories 95% of the time. When 50 laboratories out of 100 have I_p values clustering around −1, say, the probable rank for each, including the laboratories in first and fiftieth place, will be nearly the same. The use of probable rank in this way should minimize unjustified anxiety caused by the shifting of ranks from sample to sample due to chance.

Computations.—The normalization system of reporting will require more computer time than the conventional system. Computer costs may be minimized in large check series by noting that, for L greater than 20, normalized deviations are approximately normal, that is, $Z' \simeq Z$. The Peizer-Pratt approximation for a Chi-square distribution, given by Ling (7, Equation 3.6), may be used to calculate I_p directly from X as defined by Equation *15*.

If $X = M - 1$

$$I_p \simeq -(1/3 + 0.08/M)/(2M - 2)^{1/2}$$

otherwise,

$$I_p \simeq d[(M-1)\ln((M-1)/X) + X - (M-1)]^{1/2}/|X-(M-1)|$$

where $d = X - M + 2/3 - 0.08/M$.

The maximum error in calculating $P_X(X)$ with this formula (not I_p) is stated to be less than 5×10^{-4} for $M = 5$.

Conclusion

The many advantages of the proposed reporting system are best visualized when it is applied to an actual check series. Table 3 gives the results of a recent sample for a modest intracompany check series on meat products. The complete report includes Z' values for each result reported, and means and standard deviations for each method. The ranks obtained by use of the conventional and proposed systems are different because the L values vary among the methods. The probable ranks show that the laboratory in first place has a performance index significantly better than only one other laboratory. On the other hand, the laboratory in tenth place could have obtained a rank as high as fourth place.

In this series, laboratories with rejected results are not ranked. The performance index (not including the rejected result) is still reported so that the laboratory can compare its performance with those obtained on previous samples. The reasoning behind this way of handling rejected results is that, if performance indices were accumulated over several samples, it would rapidly reward a laboratory for diligent effort in finding and eliminating the cause of the apparent gross error.

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MYCOTOXINS

Determination of Aflatoxins, Ochratoxin A, and Zearalenone in Mixed Feeds, with Detection by Thin Layer Chromatography or High Performance Liquid Chromatography

MARY V. HOWELL and PHILIP W. TAYLOR
R.H.M. Research, Lord Rank Research Centre, Lincoln Road, High Wycombe, Buckinghamshire, United Kingdom HP12 3QR

A sensitive, reliable, and economical method for the determination of 6 mycotoxins in mixed feeds is described. The feed is extracted with chloroform-water and the extract is cleaned up by using a disposable Sep-Pak silica cartridge. The procedure requires less time (15 min from sample extraction to extract preparation) and less solvent (approximately one-tenth) compared with conventional methods and is suitable for a fast, economical screen. Additional cleanup procedures, involving dialysis or extraction into base, are described for samples containing high levels of interfering compounds. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) with fluorescence detection are described for identification and estimation of mycotoxins. The method has been applied to a wide range of mixed feeds, including laboratory animal diets, and raw materials. The limit of detection is 1 μg/kg for all mycotoxins measured by HPLC.

Multimycotoxin screening methods for mixed feeds, with detection by thin layer chromatography (1, 2), have been reported, together with methods for the detection of mycotoxins by high performance liquid chromatography (HPLC) in raw materials (3-6) and to a lesser extent in mixed feeds (7, 8). However, a rapid multimycotoxin preparation method with the choice of sensitive TLC or HPLC end detection has not been described for raw materials or mixed feeds.

There are 2 main objectives in the determination of mycotoxins in raw materials and mixed feeds: first, to confidently establish the absence of mycotoxins to very low levels in laboratory animal diets, as specified by agencies such as the U.S. Food and Drug Administration or the U.S. Environmental Protection Agency (both specify less than 5 μg total aflatoxins/kg); second, if mycotoxins are present, for example in animal feed, to accurately and quantitatively confirm their identity. With these objectives in mind, an economical, sensitive, and reliable method suitable for screening a wide variety of commodities by TLC with the choice of using HPLC for improved sensitivity and confirmation has been developed for aflatoxins, ochratoxin A, and zearalenone.

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Initial sample extraction with good recovery for all mycotoxins is achieved with chloroform-water. The 3 groups of mycotoxins can then be quickly separated and the extracts can be cleaned up in only 15 min with low volumes of solvent, by using a disposable Sep-Pak silica cartridge. A further purification step for zearalenone involving extraction into alkali (3, 9) improves its detection by TLC from 200 to 10 μg/kg in mixed feeds. Similarly, the determination of aflatoxin in laboratory animal diets containing grassmeal is improved if the dialysis cleanup described by Patterson and Roberts (1) is carried out on the aflatoxin fraction from the Sep-Pak cartridge. The determination of ochratoxin A by TLC is improved by using 2 solvent systems running in the same direction.

HPLC analysis of all the mycotoxins is performed on the extracts prepared for TLC. Zearalenone is determined by HPLC by using a reverse phase column with a mobile phase of acetonitrile-0.1% orthophosphoric acid and fluorescence detection. The aflatoxins are separated by HPLC using a silica column with a mobile phase of toluene-ethyl acetate-formic acid and fluorescence detection. Ochratoxin A is measured by fluorescence detection after separation on a reverse phase column with HPLC conditions similar to those described by Osborne (10) for the analysis of flour and bakery products. Limit of detection of the method is 1 μg/kg for each of the 6 mycotoxins in a mixed feed sample.

METHOD

Apparatus

(a) *Wrist-action shaker.*—Griffin flask shaker (Griffin and George Ltd, London, UK).

(b) *Chromatographic columns.*—400 × 22 mm id with sintered glass base and PTFE stopcock.

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(c) *Shaker for tubes.*—Whirlimixer (Fisons Scientific Apparatus, Loughborough, LE11 ORG, UK).

(d) *UV illumination equipment.*—365 and 254 nm. C-70 Chromato-Vue Cabinet (Ultra-Violet Products Inc., San Gabriel, CA 91778).

(e) *Liquid chromatograph.*—Model 750/03 pump (A.C.S. Ltd, Concorde St, Luton, UK), Rheodyne 7120 injector with 40 μL loop (fill by injection of 50 μL sample), and Model 3000 fluorescence spectrometer fitted with 25 μL LC flow cell (Perkin-Elmer Ltd, Beaconsfield, HP9 1QA, UK). Other suitable equipment can be substituted.

(f) *HPLC columns.*—Stainless steel, 250 × 4.6 mm id, packed with 5 μm Partisil 5 (Whatman Ltd, Springfield Mill, Maidstone, ME14 2LE, UK), or 5 μm Spherisorb ODS (Phase Separations Ltd, Queensferry, CH5 2LR, UK).

(g) *Silica cartridges.*—Sep-Pak silica cartridge (Waters Associates, Inc., Milford, MA 01757).

(h) *TLC Plates.*—20 × 20 cm plastic sheets pre-coated with silica gel G without fluorescent indicator (Polygram SL 9, Camlab, Cambridge, UK).

(i) *Dialysis tubing.*—Visking size 1-8/32 (Medicell International Ltd, 49 Queen Victoria St, London, EC4N 4SA, UK).

Reagents

Use AnalAr grade chemicals. Caution: Chloroform is a possible carcinogen.

(a) *Solvents.*—Ethyl ether (sodium-dried). HPLC grade ethyl acetate and acetonitrile. Glass-distilled grade toluene (Rathburn Chemicals).

(b) *Orthophosphoric acid.*—0.1%.—Dilute 1 mL orthophosphoric acid (sp. gr. 1.75) to 1 L with distilled water and pass solution through 0.45 μm filter.

(c) *Silica gel for column chromatography.*—Silica gel 60, 0.063–0.2 mm (E. Merck, Darmstadt, GFR), 1% moisture.

(d) *Mycotoxin standards.*—Makor Chemicals Ltd, Jerusalem, Israel. *Aflatoxin.*—5 μg B_1, G_1/mL; 1.5 μg B_2, G_2/mL. Available in solution in benzene-acetonitrile (98 + 2). *Ochratoxin A.*—10 μg/mL. Dissolve solid in benzene-acetonitrile (98 + 2). *Zearalenone.*—Prepare 2 standards by dissolving solid in methanol to give (*1*) 10 μg/mL, and (*2*) 100 μg/mL.

Extraction

Weigh 50 g finely ground feed into 500 mL round-bottom flask and add 250 mL $CHCl_3$ followed by 25 mL distilled water. Secure flask with polyethylene stopper and shake 30 min with wrist-action shaker. Filter extract through 10 g Celite 545 filter aid in folded GF/A paper (15 cm diameter) in conical funnel. Collect two 50 mL portions of filtrate. Spike one portion with 10 μL aflatoxin standard (5 μg B_1, G_1/mL; 1.5 μg B_2, G_2/mL), 20 μL zearalenone standard (10 μg/mL), and 10 μL ochratoxin A standard (10 μg/mL), giving an effective concentration of 5 μg B_1/kg, 20 μg zearalenone/kg, and 10 μg ochratoxin A/kg in the original feed. Evaporate both fractions to near dryness by using rotary evaporator.

Column Chromatographic Cleanup

Using 2 cm id column with sintered glass base and PTFE stopcock, prepare column by layering 5 g Na_2SO_4, 10 g silica gel 60, and 15 g Na_2SO_4 in $CHCl_3$. Draw off solvent to top of Na_2SO_4. Add sample to column with three 1 mL portions of toluene, washing out flask with toluene and drawing sample onto column.

Wash column with 150 mL toluene and elute zearalenone with 250 mL toluene–acetone (95 + 5) (fraction I). Wash column with 150 mL ethyl ether–hexane (3 + 1). Elute aflatoxins with 150 mL $CHCl_3$–methanol (97 + 3) (fraction II). Elute ochratoxin A with 200 mL toluene–acetic acid (9 + 1) (fraction III). Evaporate fractions I, II, and III from column to near dryness and transfer to small vials with $CHCl_3$.

Sep-Pak Cleanup

Alternatively, use Sep-Pak silica cartridge in place of column cleanup. These cartridges are inexpensive, are disposable, and greatly reduce volume of solvent needed to perform cleanup with conventional column.

Following manufacturer's instructions for use, add sample to cartridge by using two 0.5 mL portions of toluene. Wash with 10 mL toluene. Elute zearalenone with 10 mL toluene–acetone (95 + 5) (fraction I) and wash with 6 mL ethyl ether–hexane (3 + 1). Elute aflatoxins with 10 mL $CHCl_3$–methanol (97 + 3) (fraction II). Elute ochratoxin A with 10 mL toluene–acetic acid (9 + 1) (fraction III). Evaporate fractions I, II, and III to near dryness, and transfer to small vials with $CHCl_3$.

Base Cleanup for Zearalenone Fraction

Depending on sensitivity required, zearalenone fraction may be either analyzed directly or subjected to further cleanup to increase sensitivity of detection.

For additional cleanup, transfer fraction I to 15

mL graduated centrifuge tube with $CHCl_3$, and carry out alkali extraction cleanup described by Möller and Josefsson (3). Transfer final extract in $CHCl_3$ to small vial for TLC or HPLC.

Dialysis Cleanup for Aflatoxin Fraction

TLC or HPLC may be performed on fraction II from most samples. However, analysis of samples that contain significant amount of interfering compounds, such as laboratory animal diets containing grassmeal, is improved if dialysis step is included.

Transfer fraction II to 30 cm 1-8/32 Visking dialysis tubing with two 1 mL portions of acetonitrile as described by Patterson and Roberts (1), but use 40 mL 30% acetone instead of 25 mL in 50 mL stoppered flask. Wash Na_2SO_4 column finally with 30 mL $CHCl_3$-methanol (97 + 3) and add wash to $CHCl_3$ already collected. Evaporate extract to near dryness and transfer in $CHCl_3$ to small vial for TLC or HPLC.

Thin Layer Chromatography

Activate TLC plates by heating 1 h at 110°C and allowing to cool in desiccator. Cut into four 10 × 10 cm plates.

(a) *Zearalenone.*—Evaporate fraction I extracts to dryness and dissolve in 20 μL $CHCl_3$. Spot 2, 6, and 10 μL of both sample and spiked extract. Co-spot the 2 μL spiked extract spot with 1 μL standard (100 μg/mL). Spot range of zearalenone standards for estimation purposes, e.g., 2.5, 3.5, 4.5, 6, 7.5, and 9 μL of 10 μg/mL standard and 1, 1.5, 2, 2.5 μL of 100 μg/mL standard. This covers range of graduated brightness and permits theoretical range of quantification from 5 to 250 μg/kg in original sample. Develop plate in $CHCl_3$-methanol (97 + 3) in lined equilibrated tank. Air-dry and observe plate under shortwave (250 nm) UV light. Zearalenone fluoresces bright blue at R_f 0.5. Compare spots from sample with spike and standards.

(b) *Aflatoxin.*—Evaporate fraction II extracts to dryness and dissolve in 250 μL benzene-acetonitrile (98 + 2). Spot two 2.5 μL and one 5 μL aliquots of sample and spiked extract. Co-spot one 2.5 μL spiked extract spot with 1 μL standard (5 μg B_1, G_1/mL; 1.5 μg B_2, G_2/mL). Spot 1 μL standard next to sample extract spots. Develop plate in $CHCl_3$-acetone (9 + 1) in unlined unequilibrated tank. Air-dry and observe plate under longwave (365 nm) UV light. Aflatoxins appear as 4 spots in order of chromatographic mobility B_1, B_2 (blue), G_1, G_2 (green) from R_f 0.4 to 0.5. Compare spots from sample with spike and standards.

(c) *Ochratoxin A.*—Evaporate fraction III extracts to dryness and dissolve in 1 mL benzene-acetonitrile (98 + 2). Spot two 10 μL aliquots of sample and spiked extract and co-spot one 10 μL spiked extract spot with 1 μL standard (10 μg/mL). Spot 1, 2, 3, 4, and 5 μL standard (10 μg/mL) for quantification. Develop plate in $CHCl_3$-methanol (97 + 3), air-dry, and then develop plate in same dimension in toluene-acetic acid (9 + 1), both in unlined, unequilibrated tanks. Air-dry and observe plate under longwave (365 nm) UV light. Ochratoxin A appears as bright blue-green spot at R_f 0.4. Compare spots from sample with spike and standards.

High Performance Liquid Chromatography

(a) *Zearalenone.*—Prepare mobile phase of acetonitrile-0.1% orthophosphoric acid (50 + 50). Set fluorescence detector to excitation 274 nm (15 nm slit), emission 440 nm (20 nm slit). Stabilize system at flow rate of 1.0 mL/min. Dilute 100 μg zearalenone/mL standard with mobile phase to give 2 μg/mL solution, and inject 50 μL. Adjust sensitivity of fluorescence detector to give ca 60–80% full scale recorder deflection. Evaporate fraction I extracts to dryness, dissolve in 0.1 mL mobile phase and inject 50 μL into system. Compare peak heights from sample and spiked extracts to calculate concentration of zearalenone in sample.

To confirm positive result, change fluorescence excitation wavelength to 316 nm and sequentially inject 50 μL of 2 μg zearalenone/mL standard followed by 50 μL sample extract. Peak height ratio 274/316 nm obtained for sample should agree within 10% of that obtained for standard zearalenone. Flush pump and column with methanol after all samples have been run.

(b) *Aflatoxin.*—Prepare mobile phase of water-saturated toluene-ethyl acetate-formic acid (85 + 25 + 5). Set fluorescence detector to excitation 365 nm (15 nm slit), emission 425 nm (20 nm slit). Stabilize system at flow rate of 2.5 mL/min. Dilute aflatoxin standard with toluene-acetonitrile (98 + 2) to give solution containing 0.2 μg B_1, G_1/mL; 0.06 μg B_2, G_2/mL. Inject 50 μL and adjust sensitivity of fluorescence detector to give ca 60–80% full scale recorder deflection for B_1 peak. Evaporate fraction II extracts to dryness, dissolve in 0.5 mL toluene-acetonitrile (98 + 2), and inject 50 μL. Compare peak heights from sample and spiked extracts to calculate concentration of aflatoxin in sample.

To confirm positive result, change fluorescence excitation wavelength to 330 nm and sequentially inject 50 μL 0.2 μg B_1, G_1/mL; 0.06 μg

B_2, G_2/mL standard, followed by 50 μL sample extract. Peak height ratio 365/330 nm obtained for sample should agree within 10% of that obtained for standard aflatoxin. Flush pump and column with ethyl acetate after all samples have been run.

(c) *Ochratoxin A*.—Prepare mobile phase of acetonitrile–0.1% orthophosphoric acid (55 + 45). Set fluorescence detector to excitation 333 nm (15 nm slit), emission 470 nm (20 nm slit). Stabilize system at flow rate of 1.0 mL/min. Evaporate 0.2 mL of 10 μg ochratoxin A/mL standard to dryness and dissolve in 20 mL mobile phase to give 0.1 μg/mL solution. Inject 50 μL and adjust sensitivity of fluorescence detector to give ca 60–80% full scale recorder deflection. Evaporate fraction III extract to dryness, dissolve in 1 mL mobile phase, and inject 50 μL. Compare peak heights from sample and spiked extracts to calculate concentration of ochratoxin A in sample.

To confirm positive result, change fluorescence excitation wavelength to 280 nm and sequentially inject 50 μL of 0.1 μg ochratoxin A/mL standard followed by 50 μL sample extract. Peak height ratio 333/280 nm obtained for sample should agree within 10% of that obtained for standard ochratoxin A. Flush pump and column with methanol after all samples have been run.

Results and Discussion

Initial work on the cleanup procedure was carried out using a normal silica column, as described in the method, before the introduction of the Sep-Pak cartridge. The cartridge has the advantage of being convenient, quicker, and requires low volumes of solvent, which is both desirable and economical. It is necessary to dialyze diets containing grassmeal because they contain high levels of fluorescent material which chromatographs around the aflatoxin spots on the TLC plates and obscures the peaks on the HPLC chromatogram. However, the recovery of aflatoxin is somewhat reduced, and so the detection limits are twice as large by TLC or HPLC for dialyzed samples compared with non-dialyzed samples. Dialysis can be performed before the cleanup but the recovery of zearalenone and ochratoxin A is poor. Zearalenone recovery can be improved if the aqueous acetone is reduced to near dryness rather than extracted with chloroform. However, dialysis still raises the detection limits of zearalenone and ochratoxin A and it is, therefore, preferable to perform dialysis only on the aflatoxin fraction from diets containing grassmeal.

Early work on the preparation of an extract containing zearalenone involved the alkali cleanup method of Mirocha et al. (9) applied to a fraction from the silica column. However, we experienced problems with emulsions, low recovery, and time-consuming handling. Later work on base cleanup as described by Möller and Josefsson (3) resulted in a faster, simpler procedure which gave excellent zearalenone recovery at the optimum pH of 8.0.

Separate TLC developing solvents, the optimum for each group of mycotoxins, are recommended. If only one sample is being analyzed at a time, the plastic plates may be cut into strips and smaller receptacles and less developing solvent may be used.

In the development of a method to separate and detect zearalenone by HPLC, the use of a reverse phase column with a methanol-water phase, as described by Ware and Thorpe (4), gave a poor zearalenone peak shape. However, addition of orthophosphoric acid to the mobile phase improved the measured column efficiency by a factor of nearly 2. Use of methanol–0.1% orthophosphoric acid (65 + 35) and acetonitrile–0.1% orthophosphoric acid (50 + 50) gave similar zearalenone retention times, but the latter produced a better separation of zearalenone from the other fluorescent components of mixed feed.

Under the HPLC conditions described, minimum detectable amounts (signal peak height twice baseline noise) of zearalenone injected were 5.0, 2.4, and 2.0 ng at excitation wavelengths of 236, 274, and 316 nm, respectively. At these wavelengths, molar absorptivities were 29 700, 13 909, and 6020, respectively. The optimum excitation wavelength depends on the energy profile of the light source in the fluorescence detector. The Perkin-Elmer 3000 spectrometer has a pulsed xenon source which has a relatively low output below 250 nm. Thus, the wavelength of the most intense peak in the zearalenone UV absorption spectrum (236 nm) was the least sensitive for fluorescence excitation. An excitation wavelength of 274 nm gave good selectivity for determining zearalenone in the majority of samples. If a fluorescence detector with a deuterium lamp light source is used, then 236 nm may be a better choice as excitation wavelength.

Figure 1 shows the HPLC chromatogram obtained from the analysis of a laboratory animal diet for zearalenone after the additional cleanup. The diet, when analyzed by TLC, gave a positive result of 10 μg/kg. From the HPLC chromato-

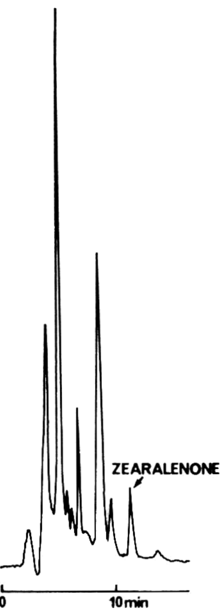

Figure 1. Chromatogram of mixed feed containing 11 μg zearalenone/kg: column, Spherisorb ODS 5 μm; mobile phase, acetonitrile-0.1% orthophosphoric acid (50 + 50); flow rate, 1 mL/min; fluorescence detector, excitation 274 nm, emission 440 nm.

gram, a zearalenone concentration of 11 μg/kg was calculated. The diet contained wheat, corn, soya, barley, fishmeal, and oatfeed. Analysis of the raw materials showed that the corn was the source of contamination. In our laboratory, routine screening of laboratory animal diets and corn plus investigatory screening of animal feeds for zearalenone is performed by TLC. Using the additional cleanup, mixed feeds containing a wide variety of ingredients, including grassmeal, have been successfully analyzed by HPLC. Analysis time was approximately 15 min per sample with a detection limit of 1 μg zearalenone/kg.

The HPLC system described by Manabe et al. (7), with a silica column and a mobile phase of toluene-ethyl acetate-formic acid-methanol (89 + 7.5 + 2 + 1.5), was evaluated for determining aflatoxin in a range of mixed feeds. Laboratory diets containing grassmeal produced chromatograms with a large broad peak which, although reduced after dialysis of the extract, still completely obscured aflatoxin G_1. Therefore, the composition of the phase was modified to water-saturated toluene-ethyl acetate-formic acid (85 + 25 + 5). The interference from grassmeal in dialyzed extracts was now eluted in the space between the peaks from aflatoxins G_1 and G_2. The modified phase also gives a better fluorescence response for aflatoxin B_1 and has the additional advantage of still being usable after storage.

Aflatoxin retention times depend on the surface area per gram of the silica column packing. It may be necessary to change the amount of toluene in the mobile phase to allow for column-to-column variations. A retention time of approximately 20-25 min for the final aflatoxin peak on the chromatogram (G_2) provides a good compromise between separation and analysis time for mixed feed. The selectivity of the mobile phase may be modified to alter the separation of a co-extractive from an aflatoxin by changing the ratio of ethyl acetate to formic acid. For instance, water-saturated toluene-ethyl acetate-formic acid in the ratio 85 + 30 + 4 will give similar retention times but a slightly different selectivity than an 85 + 25 + 5 mixture.

One factor that affects the sensitivity of fluorescence detection is the background fluorescence from the mobile phase. The fluorescence from toluene of various grades and from several suppliers was measured. The level from Rathburn's distilled grade was the lowest by a considerable margin, and was comparable to that measured for the ethyl acetate and formic acid used. The minimum detectable amounts of aflatoxin injected onto the liquid chromatograph were 0.26 ng B_1, 0.16 ng B_2, 0.28 ng G_1, and 0.12 ng G_2.

In our laboratory, routine screening of laboratory diets and corn, plus investigatory screening of animal feed for aflatoxin, is performed by HPLC. Figure 2 shows the chromatograms from (a) a laboratory animal diet, and (b) the same diet spiked before extraction with 5 μg B_1, 5 μg G_1, 1.5 μg B_2, and 1.5 μg G_2/kg. The diet was shown to contain less than 0.4 μg B_1 and G_1/kg and less than 0.2 μg B_2 and G_2/kg; these figures were typical of the detection limits obtained.

A set of conditions similar to that described by Osborne (10) was used to determine ochratoxin A by HPLC, except that a column packed with 5 μm Spherisorb ODS (C_{18}) was used instead of one packed with 10 μm LiChrosorb RP8 (C_8). As a result, the capacity factor (3.6) and column efficiency (3910) measured for ochratoxin A at 1.0 mL/min were higher. The minimum detectable amount of ochratoxin A under these conditions was 0.05 ng. Column efficiency at a flow rate of 2.0 mL/min (2390) was adequate for the analysis of most samples.

A range of mixed feeds containing a variety of

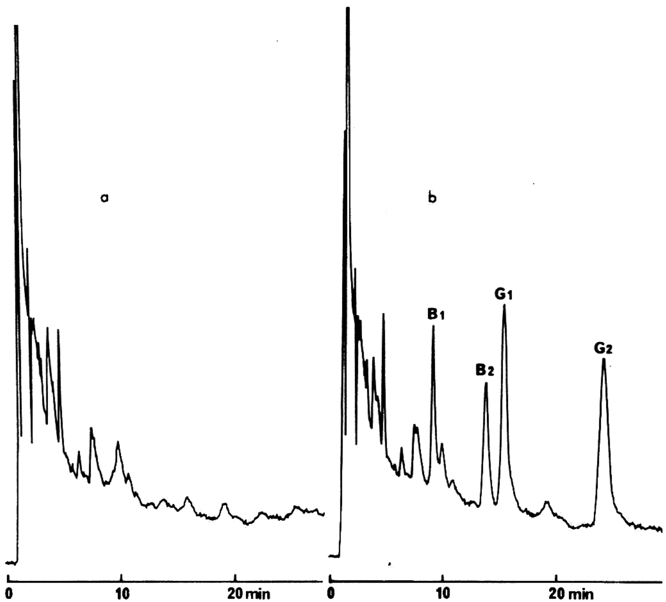

Figure 2. Chromatograms of (a) laboratory animal diet, and (b) laboratory animal diet spiked with 5 μg B_1, 5 μg G_1/kg, 1.5 μg B_2, and 1.5 μg G_2/kg. Column, Partisil 5 μm; mobile phase, water-saturated toluene-ethyl acetate-formic acid (85 + 25 + 5); flow rate, 2.5 mL/min; fluorescence detector, excitation 365 nm, emission 425 nm.

ingredients has been successfully analyzed for ochratoxin A, using a final determination by HPLC. Figure 3 shows the chromatogram obtained from the analysis of a mold-spoiled cow feed which was found to be positive by TLC and quantitated at 50 μg/kg. From the HPLC chromatogram, an ochratoxin A concentration of 45 μg/kg was calculated. The presence on the chromatogram of peaks with retention times significantly longer than that of ochratoxin A was unusual. Analysis time, using a flow rate of 2 mL/min, was normally less than 10 min and the detection limit of the method, with the final determination by HPLC, was approximately 1 μg ochratoxin A/kg.

Experiments adding mycotoxin standards to the sample before extraction showed that the recovery into the extractant from animal feed is 100%, and toxin is only lost during the cleanup procedure. This would not necessarily apply to naturally contaminated material, where initial mycotoxin concentration and hence the recovery by the extractant can only truly be estimated by determining the biological activity of the toxin in the sample.

The method specifies spiking the initial extract because this saves time and solvent and provides

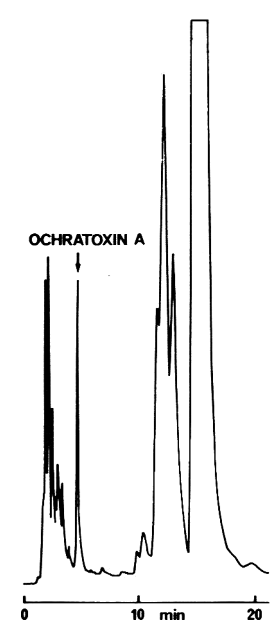

Figure 3. Chromatogram of mixed feed containing 45 μg ochratoxin A/kg: column, Spherisorb ODS 5 μm; mobile phase, acetonitrile-0.1% orthophosphoric acid (55 + 45); flow rate, 2 mL/min; fluorescence detector, excitation 333 nm, emission 470 nm.

Table 1. Typical recoveries, after cleanup, of mycotoxins added to mixed feed [a]

Mycotoxin	Added, μg/kg	Av. rec. and range, %		
		Sep-Pak [b]	Sep-Pak + dialysis [c]	Sep-Pak + base
Aflatoxin B_1	5	70 (52–104)	33 (25–44)	—
Aflatoxin B_2	1.5	83 (59–107)	47 (39–57)	—
Aflatoxin G_1	5	69 (45–86)	35 (26–48)	—
Aflatoxin G_2	1.5	79 (55–103)	43 (34–57)	—
Zearalenone	20	—	—	90
Ochratoxin A	10	90	—	—

[a] Includes laboratory animal diets plus cattle, horse, pig, chicken, and turkey rations.
[b] Means and ranges are for aflatoxin recovery from 13 different samples of laboratory diet.
[c] Means and ranges are for aflatoxin recovery from 6 different samples of laboratory diet containing grassmeal.

2 uniform extracts for comparison. If commodities other than animal feed ingredients are to be extracted, then spiking the original dry sample is recommended, until the percentage recovery into the extractant is established.

Table 1 gives the percentage recovery of the mycotoxins after the described cleanup, as measured by HPLC. The recoveries quoted for the aflatoxins were an average of 13 different samples of laboratory diet with the Sep-Pak cleanup and 6 different samples of laboratory diet containing grassmeal with the Sep-Pak plus dialysis cleanup. The samples were analyzed over a period of 4 weeks. Note the spread of recovery figures at the low spiking levels employed (5 μg B_1, G_1/kg; 1.5 μg B_2, G_2/kg). When samples were spiked at higher aflatoxin levels, more consistent recovery data were obtained. The variable recovery experienced with the Sep-Pak procedure is obviously a problem, however, the preparation of sample and spiked extracts at the same time, followed by calculation of the mycotoxin concentration by comparison of the 2 chromatograms (as described in the method), minimizes the effect of recovery variations on the final result. This, together with the speed and economy of the Sep-Pak cartridge, makes its use advantageous when screening large numbers of samples. If a specific analysis of a sample containing a low level of aflatoxin is required, column chromatography is recommended. The limits of detection for all mycotoxins by HPLC and by TLC are given in Table 2.

The method described has been applied to the routine screening of raw materials and laboratory animal diets and investigatory screening of mixed feeds. Over 300 samples have been analyzed and some have been contaminated. Aflatoxin was mainly found in mixed feeds containing groundnut or maize. Zearalenone was identified in 15 of 20 batches of maize analyzed, and in feeds containing maize. Ochratoxin A was identified in mixed feeds visibly spoiled by mold due to bad storage.

Table 2. Detection limits, μg/kg, of method applied to mixed feeds

Sample	Aflatoxin B_1		Aflatoxin B_2		Aflatoxin G_1		Aflatoxin G_2		Zearalenone		Ochratoxin A	
	TLC	HPLC	TLC	HPLC	TLC	HPLC	TLC	HPLC	TLC	HPLC	TLC	HPLC
Mixed feeds [a]	3	0.4	0.9	0.2	3	0.4	0.9	0.2	200	—	10	1
Mixed feeds with grassmeal	15	—	5	—	15	—	5	—	600	—	10	1
Mixed feeds with grassmeal, after dialysis cleanup	10	0.8	3	0.4	10	0.8	3	0.4	—	—	—	—
Mixed feeds, after base cleanup	—	—	—	—	—	—	—	—	10	1	—	—
Mixed feeds with grassmeal, after base cleanup	—	—	—	—	—	—	—	—	30	1	—	—

[a] Includes laboratory animal diets plus cattle, horse, pig, chicken, and turkey rations.

Addendum

Since preparing the manuscript, the authors have investigated an additional method of confirming positive results for aflatoxins B_1 and G_1. The addition of 50 μL trifluoroacetic acid to the sample extract (evaporated to dryness or dissolved in toluene–acetonitrile (98 + 2)) converts aflatoxin B_1 to the hydrated derivative B_{2a} and G_1 to G_{2a} (11). The derivatives B_{2a} and G_{2a} have longer retention times under the HPLC conditions described than do B_1 and G_1, respectively. The elution order observed after derivatization is B_{2a}, B_2, G_{2a}, G_2. Comparison of the HPLC chromatograms from a contaminated sample extract, before and after the addition of trifluoroacetic acid, will show the disappearance of the B_1 and G_1 peaks and the appearance of B_{2a} and G_{2a} peaks.

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Gas Chromatography with Electron Capture and Mass Spectrometric Detection of Deoxynivalenol in Wheat and Other Grains

PETER M. SCOTT, PUI-YAN LAU, and SHRINIWAS R. KANHERE
Health and Welfare Canada, Health Protection Branch, Food Directorate, Ottawa, Ontario, Canada K1A 0L2

A method for determining deoxynivalenol (DON) in wheat has been developed in conjunction with an assessment of contamination in Canada. The sample is extracted with methanol-water; the extract is treated with 30% aqueous ammonium sulfate solution and extracted with 4 portions of ethyl acetate. After further cleanup by column chromatography, the sample extract is derivatized with *N*-heptafluorobutyrylimidazole, and the DON tris-heptafluorobutyrate is determined by gas-liquid chromatography with electron capture detection. Mass spectrometric single ion monitoring at m/z 884 is used for confirmation. Detection limits are ≤0.01 μg DON/g and recoveries from wheat, using the proposed method, averaged 72 and 80% in 2 different laboratories, with coefficients of variation of 10.2 and 10.0%, respectively. The method is also applicable to determining DON in barley and corn and T-2 toxin in wheat. Virtually 100% contamination by DON of the 1980 Ontario white winter wheat and Quebec red spring wheat crops was found, based on 72 analyses made in this laboratory, but western Canadian wheat contained little or no DON.

In the summer of 1980, the Ontario white winter wheat crop was extensively contaminated with pink kernels, later shown to be associated with the presence of *Fusarium graminearum* (G. A. Neish and H. Cohen, Agriculture Canada). Analysis of an initial 25 samples of the wheat in this laboratory for trichothecenes revealed the presence of deoxynivalenol (DON; vomitoxin) in all samples; T-2 toxin, HT-2 toxin, and diacetoxyscirpenol did not appear to be present.

DON was first isolated in Japan and the United States from barley and corn infected in the field with *F. graminearum* (*F. roseum*) (1–3). The alternative name vomitoxin refers to its property of causing vomiting (emesis) in swine (3, 4). Feed refusal and associated decreased gain or even weight loss are additional effects produced by DON in animals (4–7). DON has been found in corn in several countries, often associated with emesis and/or refusal in swine, and including corn eaten by humans (3, 8–16). It has also been detected in normal-appearing samples of Japanese barley and wheat (17). Faced with this considerable evidence for the natural occurrence of DON in grains and with concern for potential contamination by trichothecenes in general, method development for these mycotoxins has been an active field. The favored means of determining trichothecenes, including DON, has been gas-liquid chromatography (GLC), with trimethylsilylation for derivative formation and with flame ionization detection (13, 18–21). Kuroda et al. (17) showed that sensitivity for the trimethylsilyl derivatives of DON and related compounds was about 2500 times better with an electron capture (EC) detector and, in fact, as little as 2 pg DON could be detected. Recently, heptafluorobutyrylation has been combined with EC determination of T-2 toxin and diacetoxyscirpenol in grains (22, 23). We have extended this reaction to DON and several other trichothecenes (P. M. Scott, S. R. Kanhere, and P.-Y. Lau, unpublished results) and, as presented in this paper, have developed an overall method for determining DON in wheat, which is being used to assess the extent of contamination in Canada and elsewhere.

The method incorporates essentially the same extraction procedure for DON as that presented by Romer and Boling (24), followed by a simple silica gel column cleanup before derivatization. Throughout the method development and application to actual samples, we frequently checked GLC results obtained by EC detection with mass spectrometric single ion monitoring (MS(SIM)) at m/z 884, the molecular ion of DON tris-heptafluorobutyrate.

METHOD

Apparatus

(a) *Gas chromatograph.*—Varian Aerograph Model 2100, or equivalent, equipped with all-glass injector, 183 cm × 2 mm id glass U-column filled with 3% OV-3 on 80–100 mesh Chromosorb W (HP), and electron capture detector (^3H/Sc foil).

(b) *High-speed blender.*—Waring, explosion-proof, with 1 L jar.

(c) *Centrifuge.*—International ¾ HP, Sorvall SS-3 automatic, or equivalent, with 250 mL bot-

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tles (use plastic bottles with Sorvall), and 5 mL glass-stopper centrifuge tubes.

(d) *Tube-shaking machine.*—Thermolyne Maxi Mix, or equivalent.

(e) *Chromatographic column.*—Glass, 27 × 1.3 cm od, with 250 mL reservoir (if preferred) and Teflon stopcock.

Reagents

(a) *Deoxynivalenol.*—Available from Myco-Lab. Co., PO Box 321, Chesterfield, MO 63107. Prepare stock solution 1 mg/mL $CHCl_3$ by warming; store in freezer. Mix well to redissolve. Evaporate 20 μL aliquot of stock solution and add 10 mL toluene-acetonitrile (95 + 5) to give 2 μg/mL working standard.

(b) *N-Heptafluorobutyrylimidazole (HFBI).*—Pierce Chemical Co., Rockford, IL 61105.

(c) *Ammonium sulfate.*—30% solution.

(d) *Diatomaceous earth.*—Hyflo Super-Cel (Fisher Scientific Co.).

(e) *Silica gel.*—For column chromatography. E. Merck silica gel 60, 0.063-0.200 mm particle size.

(f) *Solvents.*—Distilled-in-glass methanol, ethyl acetate, methylene chloride, toluene, acetone, acetonitrile, *n*-hexane.

(g) *Laboratory film.*—Parafilm 'M' (American Can Co., Greenwich, CT 06830).

(h) *Silyl-8.*—Pierce Chemical Co.

Sample Preparation

Grind 5-10 lb sample 4-5 min in Hobart vertical cutter mixer Model 40 (or equivalent).

Extraction and Cleanup

Blend 50 g sample 5 min at high speed with 250 mL methanol-water (1 + 1). Transfer mixture to 250 mL centrifuge bottles and centrifuge 5 min at 3500 rpm. Decant centrifugate and transfer 60 mL portion to 500 mL beaker. Add 240 mL 30% aqueous ammonium sulfate, mix, add 100 mL diatomaceous earth, and stir 2 min on magnetic stirrer. Filter through Whatman No. 4 fluted paper or through sintered-glass funnel (with suction). Collect 200 mL filtrate and transfer to 500 mL separatory funnel. Extract with four 100 mL portions of ethyl acetate and dry combined extracts by stirring 10 min on magnetic stirrer with 35-40 g anhydrous Na_2SO_4 for ca 10 min. Add 10-20 g more Na_2SO_4 until last portion does not form lumps. Decant through glass wool plug into 500 mL pear-shaped flask and evaporate to dryness on rotary evaporator at water bath temperature of ca 55°C. Rinse Na_2SO_4 with two 50 mL portions of ethyl acetate, add rinses to evaporating flask, and evaporate to dryness. Transfer residue to 4 mL Teflon-lined screw-cap vial with ethyl acetate and evaporate to dryness under gentle stream of nitrogen on aluminum block heated to ca 50°C.

Column Chromatography

Prepare silica gel column by adding 1 g anhydrous Na_2SO_4 onto glass wool plug at bottom of column filled with toluene. Slowly add 2 g silica gel and cap with 1 g anhydrous Na_2SO_4. Drain solvent to top of upper Na_2SO_4 layer. Dissolve sample extract in 0.5 mL methylene chloride, using tube-shaking machine, and transfer to top of column with Pasteur pipet. Rinse vial with further 0.5 mL methylene chloride and add rinse to column. Drain to top of upper Na_2SO_4 layer. Wash column with 30 mL toluene-acetone (95 + 5), ca 2 mL of which is used to rinse vial, and discard eluate. Elute trichothecenes with 50 mL methylene chloride-methanol (95 + 5) into 250 mL round-bottom flask and evaporate eluate to dryness on rotary evaporator at water bath temperature of ≤60°C. Transfer residue to 4 mL vial with methylene chloride, evaporate solvent under nitrogen, and add 4 mL toluene-acetonitrile (95 + 5). This solution contains 2 g sample equivalent/mL.

Derivatization

Remove sufficient HFBI for derivatizations and place 50 μL in each 5 mL glass-stopper centrifuge tube. Reseal bottle of HFBI with Parafilm. To the 50 μL HFBI, add 500 μL sample or standard solution (2 μg/mL) in toluene-acetonitrile (95 + 5). Seal stopper with Parafilm and mix well 1 min on tube shaker. Heat tubes 1 h in sand bath maintained at 60°C. Cool to room temperature and mix again ca 1 min. Add 1 mL 5% aqueous sodium bicarbonate solution, mix 2 min on tube shaker, and let stand to separate layers. Transfer 50 μL (≡ 0.1 g) organic layer with syringe to 2 mL vial containing 950 μL *n*-hexane. Prepare standard solution containing 0.025 μg deoxynivalenol/mL by dilution with *n*-hexane.

Gas Chromatography

If possible, carry out analysis on same day as derivatization, using following conditions: column 170°C; injector and detector 225°C; nitrogen carrier gas at 52 mL/min; attenuation 8×10^{-10} amp/mV (32×10^{-10} amp/mV with column temperature of 200°C used in developmental work); chart speed 0.25 in./min.

Depending on detector sensitivity, inject suitable amounts of standard directly onto column to obtain standard curve (5–50 pg should be attainable). Detector response (peak height) varies linearly with amount injected. Standard curve must be prepared each day. Initially inject 1–2 μL sample solution and adjust volume injected or dilute sample solution, if necessary, with *n*-hexane. Retention time for DON is 5–6.5 min. Allow at least 20 min between injections, made in duplicate for each sample.

Calculate DON concentration (μg/g) in sample and correct for method recovery. Make determinations only for sample peaks with same peak width (at half height) as standard.

Other Trichothecenes (Recovery Experiments)

For determination of diacetoxyscirpenol and T-2 toxin and detection of HT-2 toxin by GLC-EC, proceed as above except final derivatized standard solutions contain 0.25 μg toxin/mL. Use GLC column temperature of 220°C and attenuations of 32×10^{-10}, 8×10^{-10}, and 64×10^{-10} amp/mV, respectively. Standard curves then range from 150 to 1000 pg and retention times are 3.5, 12.2, and 5.7 min, respectively.

Gas Chromatography-Mass Spectrometric Single Ion Monitoring

DON.—Initially inject 5 μL sample solution into Varian Aerograph Model 1400 gas chromatograph equipped with 183 cm × 2 mm id glass column packed with 3% OV-3 on 80–100 mesh Chromosorb W (HP) and coupled via a dual stage all glass Watson-Biemann separator to Varian MAT Model 311A mass spectrometer. Adjust helium flow rate (45–60 mL/min) to give ion source pressure of ca 10^{-6} torr. Use injector temperature 215–235°C, column temperature 180°C, separator temperature 210–240°C, transfer line temperature 205–240°C, and the following mass spectrometer settings: ion source temperature 215–245°C, electron energy 70 eV, emission current 2000 μA, electron multiplier voltage 2.25 kV. Operate mass spectrometer in specific ion monitoring mode for m/z 884 (molecular ion of DON tris-heptafluorobutyrate) at resolution of 950–1000 (10% valley). Use output range 0.03 V (0.01 V, if necessary) and chart speed 1 cm/min. Quantitate observed peak at retention time of DON standard (3.2–3.6 min) by peak height measurement with reference to linear standard curve (25–150 pg at range 0.03 V; 10–75 pg at 0.01 V).

Other trichothecenes.—Use GLC column temperatures of 210, 220, and 240°C for diacetoxyscirpenol, HT-2 toxin, and T-2 toxin, respectively, with single ion monitoring at m/z 502 (molecular ion of diacetoxyscirpenol heptafluorobutyrate—HOAc), 654 (HT-2 toxin bis-heptafluorobutyrate—HOAc—$(CH_3)_2CHCH_2COOH$), and 602 (T-2 toxin heptafluorobutyrate—HOAc). Retention times are ca 4.4 min. At 0.03 V range setting, amounts of standard injected are 50–150 pg, 500–2000 pg, and >1 ng diacetoxyscirpenol, HT-2 toxin, and T-2 toxin, respectively.

Results and Discussion

The proposed method for determining DON in wheat was developed and tested during investigations on Canadian wheat in 1980. During this time, 3 variations in the partition steps were studied using a GLC column temperature of 200°C: Method A included an additional 50 mL *n*-hexane wash of the 200 mL methanol-aqueous ammonium sulfate filtrate and the use of four 100 mL portions of ethyl acetate-methylene chloride (1 + 1) to extract this filtrate; method B was the proposed method, i.e., no hexane wash and ethyl acetate for extraction; and method AB was the same as method A with omission of the hexane wash. Although method A performed well with wheat samples from Ontario and Quebec (Table 1), neither the hexane wash nor the use of the lower polarity extraction solvent, steps which greatly increased method time because of emulsions, was of any value in removing an interference found in western Canadian wheat (Table 1) and it was necessary to lower the GLC column temperature to 170°C to effect separation. However, because much of the earlier work on Ontario and Quebec samples was carried out with method variation A and a GLC column temperature of 200°C, GLC-EC and GLC-MS(SIM) analyses on extracts obtained using this method variation are included in Table 1 to illustrate that in nearly all these cases good agreement could be obtained using the 2 means of quantitation. It is likely that the proposed method as written (column temperature 170°C) would also have produced similar agreement, as seen with some samples in Table 1 and depicted in Figures 1 and 2.

Filtration of the initial methanol-water extract after centrifugation was originally included in the method. This step was unnecessary and did not affect recoveries of DON from wheat, which ranged from 54 to 83% as measured by GLC-EC (column temperature 170°C) and from 55 to 94% by GLC-MS(SIM) (Table 2). The mean recovery of DON from ground wheat determined in this

Table 1. Comparison of DON found in naturally contaminated wheat samples by GLC–EC and GLC–MS(SIM), using the proposed method with minor variations

Origin of sample	DON,[a] µg/g	
	GLC–EC[b]	GLC–MS(SIM)
METHOD A[c]		
Western Canada (flour)	ND (<0.01)	ND (<0.007)
Ontario	0.33[d]	0.28
	0.31	0.36
	0.81	0.89
	2.63[d]	2.24
	0.35[d]	0.14
	0.77[d]	0.80
	0.61[d]	0.60
	0.33	0.31
	0.16	0.14
	0.072	0.050
	0.92	0.66
Quebec	0.19	0.17
	0.41	0.45
	0.27	0.21
	0.15	0.040
Ontario (1979)	0.007	0.015
	ND (<0.004)	0.004
	0.064	0.093
	0.044	0.060
Quebec	0.83	0.83
	0.29	0.33[e]
	0.89	0.92[e]
	0.28	0.31
	0.31	0.28[e]
	0.44	0.44[e]
	0.065	0.063[e]
METHOD B[c]		
Ontario	3.25	2.65
	0.95	0.75
Alberta	0.071	0.018
	0.14	ND (<0.003)
	0.006	ND (<0.003)
Saskatchewan	0.049	ND (<0.003)
Saskatchewan		
Detn 1	0.15	
Detn 2	ND (<0.01)[f]	0.004
Saskatchewan	0.063	ND (<0.003)
	0.091	0.003
	trace	0.004
Manitoba	0.073	ND (<0.003)
	0.004	ND (<0.003)
Ontario	0.75[f]	0.62
	0.063[f,g]	0.039
Quebec	0.92[f]	0.84
	5.28[f]	5.27
Quebec[h]	2.38[f]	2.45
Western Canada[h]	0.027[f]	0.026

[a] Without correction for recoveries.
[b] Column temperature 200°C except where noted.
[c] See text for method variations.
[d] Single standard injection only.
[e] Analysis of 6-day-old diluted derivative solution.
[f] Column temperature 170°C.
[g] Wide peak.
[h] Extract supplied by J.-P. Hanchay, Health Protection Branch, Quebec Region, who found 2.0 and 0.03 µg DON/g, respectively, in the Quebec and western Canadian samples.

laboratory over a period of 2 months, using the proposed method, was 72% ($n = 14$, CV 10.2%) by GLC-EC and 74% by GLC-MS(SIM) ($n = 12$, CV 15.3%) at spiking levels of 0.1–1.0 µg/g. Recoveries carried out independently in a second laboratory over a 3 day period (E. Tarter, Health Protection Branch, Ontario Region, unpublished results) at a spiking level of 0.2 µg DON/g wheat averaged 80% ($n = 10$, CV = 10.0%); the GLC column temperature was 175°C and attenuation was 16×10^{-10} amp/mV. Two factors affecting recoveries were studied: (*1*) Re-extraction with methanol-water of ground wheat spiked with 1

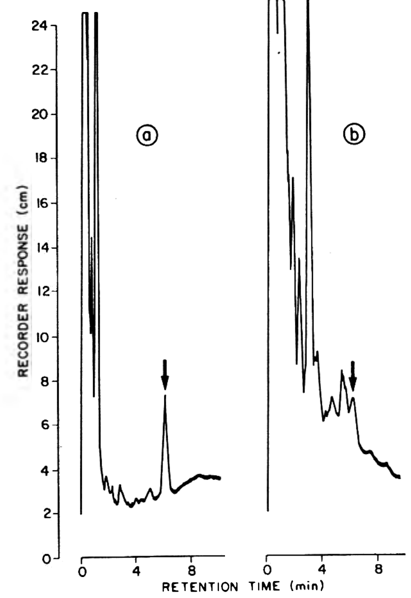

Figure 1. Gas chromatograms of extracts of naturally contaminated (a) Ontario white winter wheat and (b) western Canadian wheat.
Column temperature 170°C, attenuation 8×10^{-10} amp/mV. Arrows indicate retention time of DON tris-heptafluorobutyrate: (a) 2 µL 0.01 g equivalent wheat/mL injected and estimated to contain 15 pg DON corresponding to 0.75 µg DON/g wheat; (b) 2 µL 0.1 g equivalent wheat/mL injected; 5 pg DON, 0.025 µg DON/g wheat.

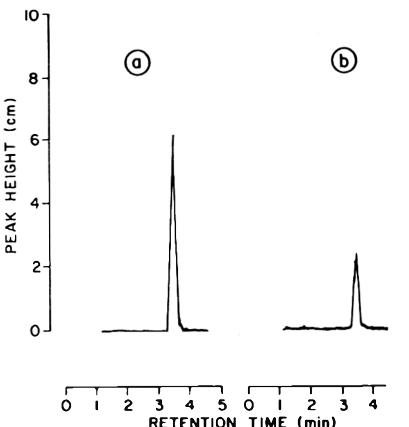

Figure 2. GLC-MS(SIM) at m/z 884 of extracts of naturally contaminated Ontario winter wheat (a) and western Canadian wheat (b) from Figure 1.

(a) 5 μL 0.01 g equivalent wheat/mL injected and estimated to contain 32 pg DON corresponding to 0.64 μg/g wheat; (b) 5 μL injected, 0.1 g equivalent wheat/mL, 13 pg DON, 0.026 μg/g wheat. Column temperature 180°C, range 0.03 V.

μg DON/g yielded only an additional 5 and 7% DON compared with 70 and 68% recovery determined by GLC-EC and GLC-MS(SIM), respectively; and (2) the column chromatographic step was tested with 2 naturally contaminated wheat samples and yielded virtually all the DON in the second fraction (Table 3).

The use of either the electron capture detector or single ion monitoring of the molecular ion of DON tris-heptafluorobutyrate permits detection and determination of very low concentrations ($\leq$0.01 μg/g) of DON in wheat (Table 1). Detector responses for standard DON varied from 2 to 6 pg/cm by GLC-EC and from 5 to 12 pg/cm by GLC-MS(SIM) at the column temperatures (170-200°C) and normal attenuations used. It should be emphasized that responses did vary, and standard curves should be made daily.

Romer et al. (22) found that T-2 toxin and diacetoxyscirpenol could be derivatized with HFBI after only 15 s at room temperature. We found that DON requires heating 1 h at 60°C. The concentrated reaction mixture was stable for at least 3 h at room temperature; however, about 40% deterioration was noted after 2 days storage, even over Na_2SO_4, by comparison with heptachlor internal standard. On the other hand, the diluted solution (0.025 μg/mL) was stable for at least 2 days. Thus, it is recommended that derivative solutions be diluted if GLC analysis on the same day is not possible.

Based on comparison of GLC-EC and GLC-MS(SIM) determinations on actual samples, the method of analysis used for determining DON in wheat is also applicable to corn, barley, and

Table 2. Recovery of DON added to wheat flour, ground wheat, barley, and corn

Sample	DON added, μg/g	Method variation[a] and column temp. for GLC-EC, °C	Recovery,[b] %	
			GLC-EC	GLC-MS(SIM)
1 (wheat flour)	0.1	A 200	72, 73	58[c], 74[c]
	1.0	A 200	85, 59	62[c], 59[c]
	0.1	B 200	86, 70	68[c], 58[c]
	1.0	B 200	78, 68	67[c], 45[c]
2 (wheat)	0.5	B 200	84, 80	
		170	78[c], 76[c]	—
3 (wheat)	0.1	B 200	101, 102	
		170	83[c], 79[c]	80, 81
	0.5	B 200	77, 88	
		170	65[c], 71[c]	64, 70
	1.0	B 200	78, 86	
		170	67[c], 74[c]	66, 69
4 (wheat)	0.1	B 170	74, 74	92, 94
	0.5	B 170	70, 75	75, 77
	1.0	B 170	66, 54	67,55
5 (wheat)	0.2	B 175	80[d]	—
6 (barley)	0.5	B 170	82, 91	—
7 (corn)	0.5	B 170	76, 79	—

[a] Method variations described in text; for Samples 3, 4, 5, 6, and 7, filtration of methanol-water extract was omitted.
[b] Duplicate recovery experiments except for Sample 5. Corrections were made for blank sample contribution, if any.
[c] Final determination on 1-2-day-old diluted derivative solution.
[d] Results from E. Tarter, Health Protection Branch, Ontario Region, n = 10, CV = 10.0%.

Table 3. Determination of DON in extracts of Ontario wheat, using 2 g silica gel column

Column fraction	DON,[a] μg/g	
	Sample 1	Sample 2
30 mL toluene–acetone (95 + 5)	ND (<0.003)	ND (<0.003)
50 mL methylene chloride–methanol (95 + 5)	3.25	0.95
20 mL methylene chloride–methanol (95 + 5)	0.014	0.008

[a] Measured by GLC–EC at 200°C column temperature, uncorrected for overall method recovery (method B).

soybeans (Table 4). Good recovery of DON added to barley and corn has been obtained (Table 2).

The method was also tested for recovery of other trichothecenes added to wheat. Nivalenol, which was present with DON in samples of wheat and barley analyzed in Japan (17), was unfortunately not carried through our procedures of extraction and cleanup. It was not detected in samples of wheat spiked with 0.1, 0.5, and 1.0 μg nivalenol/g, although the standard was detectable in amounts comparable with DON, with a retention time for the major peak of 3.8 min (DON 6.2 min). However, recoveries of diacetoxyscirpenol and T-2 toxin were satisfactory (Table 5). HT-2 toxin could also be detected by our method but because the standard deteriorated by over half throughout the day relative to diacetoxyscirpenol, quantitation was not reliable, particularly if all 3 toxins were determined on one day in the same derivative solution. Detection of T-2 toxin by GLC-MS(SIM) at m/z 602 was not sensitive; the limit was about 1 ng (compared with 13–23, 40–80, and 1–3 pg for diacetoxyscirpenol, HT-2 toxin, and DON, respectively, at a signal-to-noise ratio of 3:1). During the initial screening of Ontario wheat samples for DON, diacetoxyscirpenol, HT-2 toxin, and T-2 toxin by GLC-EC, using method A and a column temperature of 200°C, a peak was observed at the retention time of diacetoxyscirpenol in 6 of 25 samples. However, GLC–MS(SIM) analysis of the 2 extracts with the highest amounts of this material (0.62 and 2.2 μg/g, measured by GLC-EC as diacetoxyscirpenol) proved negative; thus there is a strong probability of finding a false positive for diacetoxyscirpenol by GLC-EC analysis only. One of the 25 samples contained a false positive for HT-2 toxin, determined as 0.23 μg/g, but was negative by GLC-MS(SIM).

In conclusion, using the proposed method or a variation, just over 100 samples of Canadian wheat were analyzed in this laboratory during

Table 4. Determination of DON in commercial corn, barley, and other products by GLC–EC and GLC–MS(SIM), using proposed method

Sample	Origin	DON,[a] μg/g		
		GLC–EC		
		170°C	200°C	GLC–MS(SIM)
Corn	Ontario		2.07	1.66
			0.74	0.63
		0.73	0.72	—
		0.45	0.45	—
		0.15[b]		0.20
		0.43[b]		0.48
		0.82[b]		1.04
		0.36		0.36
		0.35		0.34
Corn	USA	0.51		0.44
Corn meal	—	0.046		0.040
Barley	Ontario	0.073[b]		0.10
Barley	western Canada	ND (<0.01)[b]		0.005
Feed barley	Ontario	ND (<0.01)[b]		0.006
		0.013[b]		0.018
Pot barley	—	ND (<0.01)[b]		ND (<0.005)
Soybeans	Ontario	ND (<0.01)[b]		ND (<0.005)
		ND (<0.01)[b]		ND (<0.005)

[a] Uncorrected for method recovery.
[b] Analysis on 1-day-old diluted derivative solution.

Table 5. Recovery of T-2 toxin, HT-2 toxin, and diacetoxyscirpenol from ground wheat

Trichothecene	Added, μg/g	Recovery,[a] %	
		GLC–EC	GLC–MC (SIM)
Diacetoxyscir-penol	0.5	89[b], 78[b]	88, 89
	1.0	78[b], 74[b]	88, 82
T-2 toxin	0.5	85, 89	ND[c]
	1.0	77, 76	ca 50[d]
HT-2 toxin	0.5	—	100, 99
	1.0	—	94, 75

[a] Duplicate recovery experiments.
[b] Corrected for small contribution from blank (ca. 0.08 μg/g).
[c] ND = not detected; detection limit 0.5 μg/g.
[d] Final determination on 1-day-old diluted derivative solution.

1980. Forty-four of 45 samples of the Ontario 1980 white winter wheat crop contained DON in concentrations of 0.01–4.3 μg/g as determined by GLC-EC and corrected for method recovery; 15 positive samples were confirmed by GLC–MS(SIM) (Table 1). There appeared to be higher levels of DON in feed-grade wheat (grade 6) compared with wheat graded 1–5: 73 and 14%, respectively, of these samples contained ≥0.3 μg/g. DON was also found in all of 7 samples of Ontario white winter wheat from the 1979 crop (grades 1–4) at levels of 0.01–0.13 μg/g (corrected for recovery) and in a single sample from 1973 (0.62 μg/g). In the Quebec red spring wheat crop, 100% contamination by DON was found, based on analysis of 27 samples, of which 13 were determined by GLC–MS(SIM) (Table 1). Eighty-five percent of these Quebec samples contained ≥0.3 μg DON/g and there was little difference in the pattern of distribution in DON levels between wheat intended (but not permitted to be used) for human consumption (0.09–1.1 μg/g, corrected) and that intended for animal consumption (0.06–7.0 μg/g, corrected). Western Canadian wheat was fortunately not a problem and of 25 samples analyzed in this laboratory none contained more than 0.08 μg/g of material measured as DON by GLC-EC, while 10 of the samples analyzed by GLC–MS(SIM) all contained <0.03 μg/g (Table 1). In addition, the proposed method has demonstrated the presence of DON in barley and food-grade corn, confirmed by GLC–MS(SIM) in most cases (Table 4). Contamination of certain Canadian grains by this trichothecene is a problem that requires further research from the viewpoint of potential harm to human health.

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AOAC

High Pressure Liquid Chromatographic Determination and Fluorescence Detection of Aflatoxins in Corn and Dairy Feeds

HUGUETTE COHEN and MICHEL LAPOINTE
Agriculture Canada, Food Production and Inspection Branch, Laboratory Services Division, Ottawa, Ontario, Canada K1A 0C5

A high pressure liquid chromatographic (HPLC) method is described for the determination of aflatoxins B_1, B_2, G_1, and G_2 in animal feeds at levels as low as 2.5 ppb. Samples are extracted with acetonitrile-water and initially purified by using a Sep-Pak silica cartridge. The aflatoxins are then reacted with trifluoroacetic acid and acetonitrile-water (1 + 1). After filtration, the aflatoxins are completely resolved on a 10 μm C_{18} column using a radial compression separation system with an acetonitrile-water solvent system. Aflatoxins G_{2a}, B_{2a}, G_2, and B_2 were reported in less than 30 min, using fluorescence detection. The method was successfully applied to samples at levels of 20-2.5 ppb added aflatoxins with recoveries in the range of 82-99%.

Aflatoxins are secondary toxic metabolites produced by certain strains of fungi such as *Aspergillus parasiticus* and *A. flavus* which develop on numerous agricultural substrates, i.e., cereal grains and oil seed, under critical environmental conditions of humidity and temperature. These metabolites are also found as contaminants in food and feed derived from the meals of these seeds (1-6). Interrelations exist between man, animals, and mycotoxin-contaminated products. The toxin can be ingested by man or can be transferred in material used to feed animals which can convert aflatoxin B_1 to a toxic and carcinogenic metabolite, aflatoxin M_1 (7). This metabolite can be found in meat and eggs (8).

Numerous methods have been published for determining aflatoxins in food or feeds. Thin layer chromatographic procedures, besides lacking accuracy, are susceptible to degradation of the aflatoxins by light and reactive vapors in air during the analysis. With the advent of high pressure liquid chromatography (HPLC) as a technique, the accuracy and precision of analysis has been tremendously improved. Initially, HPLC methods used normal phase silica gel (5-10 μm) adsorption systems to resolve individual aflatoxins (7-10). However, background interferences are high and subsequent injections are delayed because of the possibility of later eluting peaks. On the other hand, the reverse phase system, when combined with the conversion of aflatoxins to their hemiacetals by trifluoroacetic acid (TFA)-catalyzed hydration of the terminal furan ring double bond (11, 12), provided a lower detection limit for fluorescence detection (11, 13-21).

The method described here involves a fast and simple purification before HPLC analysis by reverse phase chromatography. Separations are performed by using a radial compression module with a 10 μm C_{18} column with acetonitrile-water as the mobile phase.

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METHOD

Apparatus

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

(b) *Liquid chromatograph.*—Equipped with M6000 pump, and U6K septumless injector (Waters Associates Inc., Milford, MA 01757).

(c) *Fluorescence detector.*—Model 970 with variable wavelength excitation and 418 nm cutoff filter (Schoeffel Instrument Corp., Westwood, NJ 07675), or equivalent.

(d) *Liquid chromatographic column.*—10 cm × 8 mm Radial-Pak 10 μm C_{18} cartridge used in conjunction with radial compression separation system (Waters Associates Inc.).

(e) *Laboratory data system.*—Model 3354 (Hewlett-Packard Co., Avondale, PA 19311).

(f) *Mixer.*—Thermolyne Maxi Mix.

(g) *Filter paper.*—7.0 cm, Whatman No. 2.

Reagents

(a) *Solvents.*—All solvents were distilled in glass (Caledon, Georgetown, Ontario).

(b) *Adsorbents.*—Sep-Pak silica gel cartridge (Waters Associates Inc.).

(c) *Mobile phases.*—Sep-Pak, benzene and chloroform-ethanol (98 + 2); Radial-Pak C_{18} column, water-acetonitrile (1.5 + 0.5).

(d) *Trifluoroacetic acid (TFA).*—PCR Research Chemicals Inc., Gainesville, FL 32602.

(e) *Aflatoxin standards.*—Dissolve aflatoxins to concentration of 50 pg/μL.

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Table 1. Recovery of aflatoxins added to animal feeds

Sample	Added, ppb	Recovery, %[a]			
		B_1 (B_{2a})	B_2	G_1 (G_{2a})	G_2
Dairy ration	20	92	99	93	99
SD		10.7	1.1	0.6	0.8
CV, %		11.6	1.1	0.6	0.8
Milking ration	10	85	88	91	90
SD		6.0	6.5	4.8	3.7
CV, %		7.0	7.4	5.3	4.1
Dairy ration	5	82	87	86	82
SD		2.5	8.3	3.5	2.2
CV, %		3.1	9.5	4.0	2.7
Dairy supplement	2.5	98	90	93	93
SD		3.9	7.2	5.1	5.2
CV, %		4.0	8.0	5.5	5.6

[a] Each recovery is the average of 3 determinations.

Extraction

Grind samples to pass 2 mm sieve. Weigh 10 g sample into 500 mL glass-stopper Erlenmeyer flask, add 200 mL acetonitrile and 30 mL water, stopper, and shake 1 h, using wrist-action shaker set at fast rate. Filter sample through fluted paper into 500 mL round-bottom flask, and rinse filter paper twice with 50 mL portions of acetonitrile. Evaporate filtrate to ca 50 mL over 50°C water bath, using rotary evaporator. Add 100 mL $CHCl_3$, shake, and transfer to 250 mL separatory funnel. Drain lower organic layer through bed of Na_2SO_4 into 300 mL round-bottom flask. Partition aqueous layer with another 100 mL $CHCl_3$ and combine with first fraction in round-bottom flask. Evaporate to dryness over 50°C water bath, using rotary evaporator. Dissolve residue in 2–3 mL benzene.

Cleanup

Attach Sep-Pak cartridge to 50 mL glass syringe. Transfer residue from round-bottom flask to syringe. Elute entire residue into Sep-Pak. Wash round-bottom flask with two 2 mL portions of benzene and quantitatively transfer to syringe, again eluting washings from round-bottom flask into Sep-Pak. Discard benzene washings from Sep-Pak. Wash round-bottom flask with 5 mL $CHCl_3$–ethanol (98 + 2), and transfer to syringe, again eluting into Sep-Pak. Add an additional 40 mL $CHCl_3$-ethanol to syringe and collect entire $CHCl_3$–ethanol fraction containing aflatoxins in 100 mL round-bottom flask. Evaporate solvent over hot water bath, using rotary evaporator, to 2 mL. Transfer aflatoxin-containing fraction quantitatively to 15 mL glass-stopper centrifuge tube. Evaporate organic layer over hot water bath to ca 100 μL with aid of gentle stream of nitrogen.

Derivatization

To oily residue from previous step, add 100 μL TFA and vortex-mix 1 min. Add 4 mL acetonitrile–water (1 + 1), and vortex-mix for another minute. Bring to final volume of 5 mL, again vortex-mixing to ensure homogeneity. Filter sample through 7.0 cm No. 2 paper. Clear filtrate is now ready for HPLC analysis.

Preparation of Standard Curve

Evaporate 10 mL aflatoxin standard to dryness using gentle stream of nitrogen; then add 100 μL TFA and vortex-mix 1 min. Dilute to 10 mL with acetonitrile–water (1 + 1). Use this derivatized standard for final analysis by HPLC. Aflatoxins B_2 and G_2 remain unaffected.

Set HPLC flow rate to 1 mL/min. Set fluorescence detector parameters as follows: excitation wavelength, 365 nm; emission filter, 418 nm; sensitivity, 5.0; range, 0.01 μA; time constant 4 s. Set recorder chart speed at 20 cm/h.

An analytical calibration curve for aflatoxins B_{2a}, B_2, G_{2a}, and G_2 was obtained daily by plotting

Table 2. Determination of aflatoxins in naturally contaminated corn samples[a]

Sample	Aflatoxin, ppb		
	B_1 (B_{2a})	B_2	G_1 (G_{2a})
1	1477 (1470)	70 (67)	74 (60)
2	130 (128)	15 (14.5)	
3	30 (25)	3.2 (3)	

[a] Numbers in parentheses are values obtained by using AOAC method **26.049–26.051** (22). No G_2 was detected.

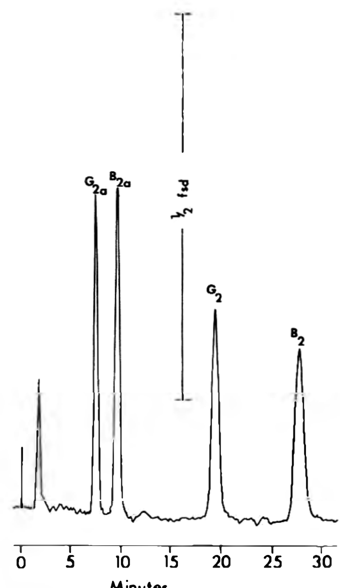

Figure 1. Chromatogram of 500 pg aflatoxins G_{2a}, B_{2a}, G_2, and B_2. See text for HPLC conditions.

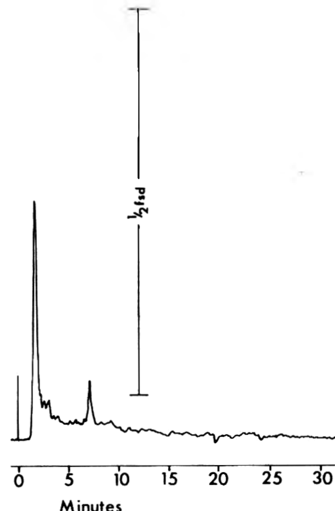

Figure 2. Chromatogram of a dairy feed sample in which no aflatoxins were detected. See text for HPLC conditions.

different amounts of aflatoxins (pg) against observed peak area.

Under above conditions, 100 pg of each aflatoxin could easily be detected with retention time of 7.31 min for G_{2a}, 9.44 min for B_{2a}, 19.13 min for G_2, and 27.35 min for B_2.

Results and Discussion

Aflatoxins G_{2a}, B_{2a}, G_2, and B_2 were completely resolved in standards and in extracts of dairy feeds. The use of the Radial-Pak 10 cm × 8 mm column reduced required operating pressure, and the larger diameter column also allowed an increase in sample size. The radial compression separation systems allowed us to increase the organic content of the mobile phase while still achieving baseline separation. The higher percentage of acetonitrile in the mobile phase compared with previously developed mobile phases prolongs column life.

The Sep-Pak silica cartridge was a quick and easy purification step for determining aflatoxins in dairy feeds. The benzene fraction eluted much of the oil from the sample. The chloroform-ethanol solvent system quantitatively recovered the aflatoxins from the Sep-Pak.

Conversion of the aflatoxins from the Sep-Pak fraction to their hemiacetals by TFA-catalyzed hydration of the terminal furan ring double bond provided a detection limit of 2.5 ppb in dairy feeds.

Before HPLC analysis, the samples were filtered through a fine grade paper. The filtrate was clear, leaving behind undissolved organic constituents on the paper. No appreciable loss of aflatoxins was detected during this step.

The aflatoxins gave a linear response from 50 to 1000 pg. Once the linear range was estab-

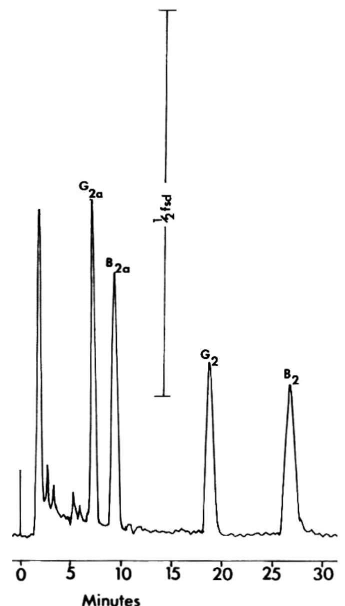

Figure 3. Chromatogram of a dairy feed sample spiked with 10 ppb aflatoxins. See text for HPLC conditions.

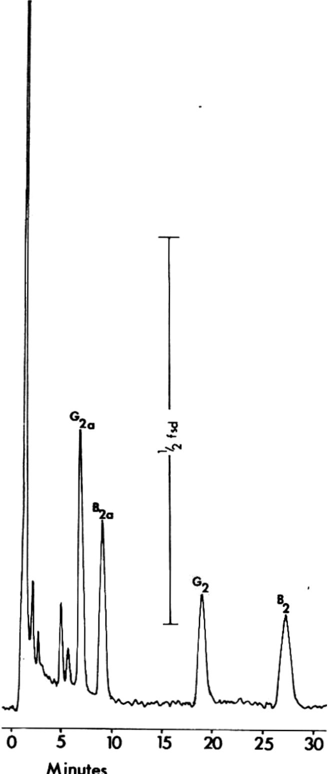

Figure 4. Chromatogram of a dairy feed sample spiked with 2.5 ppb aflatoxins. See text for HPLC conditions.

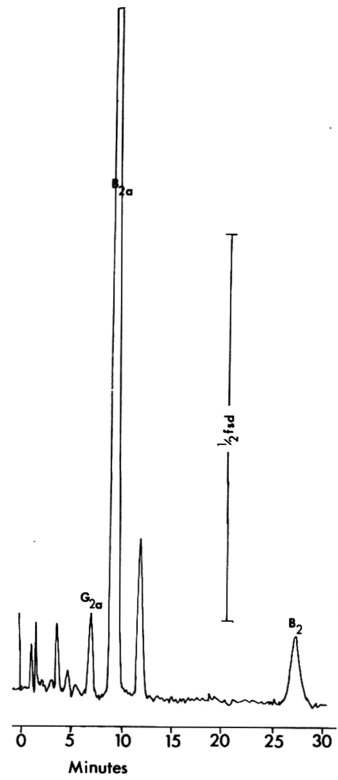

Figure 5. Chromatogram of a corn sample showing 1477 ppb B_1 (B_{2a}), 70 ppb B_2, and 74 ppb G_1 (G_{2a}). See text for HPLC conditions.

lished, aflatoxin levels in samples over the range of the calibration curve could be easily estimated.

Representative recoveries of aflatoxins added to dairy feeds at levels of 20–2.5 ppb are shown in Table 1. All samples were done in triplicate along with a separate blank. Recoveries ranged from 82 to 99%.

Table 2 gives results obtained on naturally contaminated field samples, showing the presence of aflatoxins in the range of 3.2–1477 ppb. Data in parentheses are results for the same samples, but obtained by using the AOAC official first action method for corn, **26.049-26.051** (22).

Figure 1 shows a typical chromatogram of 500 pg each of aflatoxins G_{2a}, B_{2a}, G_2, B_2. Figure 2 is a chromatogram of a dairy feed sample before being spiked with aflatoxins, Figure 3 is a chromatogram of dairy feed sample spiked at 10 ppb, Figure 4 is a chromatogram of dairy feed sample spiked at 2.5 ppb aflatoxins. Figure 5 is a chromatogram of a corn sample showing 1477 ppb B_1 (B_{2a}), 70 ppb B_2, and 74 ppb G_1 (G_{2a}).

In conclusion, the method described allowed detection of aflatoxin in dairy feeds at levels as low as 2.5 ppb. There were no discernible interferences in the final analysis by HPLC. The radial compression separation system operated in a reproducible manner with long column life.

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High Pressure Liquid Chromatographic Determination of Aflatoxins in Spices

MICHAEL J. AWE and JOHN L. SCHRANZ
Stange Co., 342 N Western Ave, Chicago, IL 60612

High pressure liquid chromatography with fluorescence detection is used to determine aflatoxin in 5 common spices. A 10 μm microparticulate silica gel column is used with a dichloromethane-cyclohexane-acetonitrile solvent system to resolve aflatoxins B_1, G_1, B_2, and G_2. The fluorescence detector contained a silica gel-packed flowcell. Samples of black, white, and red pepper, ginger, and nutmeg were extracted according to a previously published method. Recoveries from aflatoxin-free samples of white pepper, ginger, and red pepper spiked with 1-50 μg aflatoxin/kg ranged from 64 to 92%.

During the last decade, the search for aflatoxins in agricultural products has expanded to spices (1-6). The analytical methods employed have included 2-dimensional thin layer chromatography (TLC) and minicolumn detection. Both techniques allow for sensitive analysis; however, recent advances in high pressure liquid chromatography (HPLC) with fluorescence detection have shown that better separation and quantitation can be obtained with this technique (7-9). Pons and Franz (7, 10, 11) reported that near baseline resolution of aflatoxins B_1, B_2, G_1, and G_2 was obtained by using a microparticulate silica gel column with a chloroform- or dichloromethane-based solvent system. Sensitive fluorescence detection was achieved without the formation of hemiacetal derivatives (8) by using a detector with the silica gel-packed flowcell first introduced by Panalaks and Scott (9).

A method of analysis for aflatoxin designed especially for spices was lacking until Suzuki et al. (1) developed one based in part on the work of Pons et al. with mixed feed (12). The method involved extraction of the ground spice with dichloromethane and both column chromatographic cleanup and lead acetate treatment of the extract. Recently, Stoloff and Trucksess (4) introduced an improved version of this method designed specifically for ginger root and ginger oleoresin. This version added 2 cleanup steps using hexane and carbon tetrachloride, performed before the column chromatographic step. This resulted in a much cleaner final extract that for the first time allowed for 1-dimensional TLC.

The purpose of this investigation is to determine if the extraction and cleanup procedure of Stoloff and Trucksess (4), combined with the HPLC separation and detection system described by Pons (11), is applicable to the determination of aflatoxins in ginger and 4 other spices. The spices analyzed were those in which a large interest has been reported and which have an incidence of aflatoxin. Flannigan and Hui (5) tested 23 different spices for the presence of aflatoxin-producing strains of *Aspergillus flavus* and found them only in ginger, white and red pepper, and Jamaican pepper. Scott and Kennedy (2) analyzed 24 samples of black and white pepper and did not find any aflatoxin; however, they did detect B_1 and traces of B_2 and G_1 in one third of the cayenne pepper and in all of the Indian chili powder analyzed. Suzuki et al. (1) analyzed 13 different spices and found aflatoxins G_1 and G_2 in 3 of 7 samples of black pepper, and B_1 and B_2 in 3 of 5 samples of nutmeg. One sample of celery seed contained 3.7 μg G_1/kg. Beljaars et al. (3) detected aflatoxin in 30 of 40 commercial nutmeg samples in concentrations of 1.0-23.2 μg B_1/kg. Aflatoxin was detected in the majority of 1978 Cochin ginger root lots analyzed by members of the American Spice Trade Association (4). Therefore, we decided to test our procedure on samples of black, white, and red pepper, ginger, and nutmeg.

The extraction and cleanup procedures are identical to those described by Stoloff and Trucksess (4), except for a slight modification of the column chromatographic step.

METHOD

Apparatus

Equipment specified is not restrictive; other suitable equipment can be substituted.

(a) *Liquid chromatograph.*—Nester-Faust Model 1210 with screw-driven constant displacement pump, septum-sealed injector, and Varian Fluorichrom fluorescence detector equipped with 7-54 and 7-60 excitation filters (360 nm) and 430 nm interference emission filter. Detector

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flowcell was purchased prepacked by manufacturer with LiChrosorb 60 silica gel (30 μm, E. Merck, Darmstadt, Germany).

(b) *Column.*—Waters Associates Porasil silica gel (10 μm), 3.9 mm id × 30 cm; 2.1 mm id × 5 cm guard column packed with LiChrosorb 60 silica gel (30 μm) was fitted between injector and analytical column.

(c) *Recorder.*—Hewlett-Packard Model 7123A, 0.32 cm/min chart speed.

(d) *Integrator.*—Hewlett-Packard Model 3370B.

(e) *Cleanup columns.*—10 mm id × 300 mm, Teflon stopcock, 100 mL reservoir (055-723, Curtin Matheson Scientific, Elk Grove Village, IL 60007).

(f) *Concentrator tube.*—4 mL (K570050, Kontes, Vineland, NJ 08630).

(g) *Septum-sealed reaction vial.*—5 mL (6604, Alltech Associates, Deerfield, IL 60015).

Reagents

(a) *Solvents.*—Reagent grade dichloromethane, cyclohexane, acetonitrile, methanol, hexane, carbon tetrachloride, benzene, anhydrous ethyl ether, absolute ethanol, chloroform, and spectro-quality methanol.

(b) *HPLC elution solvent.*—Prepare from water-saturated dichloromethane–cyclohexane–acetonitrile (25 + 7.5 + 1.0) with 0.5% added absolute ethanol (7). Amount of ethanol needed to obtain optimum resolution can be varied to compensate for differences among silica gel columns (11).

(c) *Silica gel.*—E. Merck 7734, 70–230 mesh. Dry 1 h at 130°C.

(d) *Aflatoxin standards.*—Prepare from individual crystalline aflatoxins (Supelco, Inc., Bellefonte, PA 16823). Dissolve ca 5 mg crystals in 4 separate 1 L flasks containing 1 L spectro-grade methanol. Determine actual concentration by measuring specific absorbance maxima for each aflatoxin between 420 and 210 nm using molar absorptivities given in secs **26.006** and **26.007** (13). Prepare HPLC standard solution by using a microliter syringe to transfer sufficient quantity of aflatoxin solutions prepared above to reaction vial to produce aflatoxin standard containing 100 ng B_1, 200 ng G_1, 20 ng B_2, and 40 ng G_2/mL. Completely evaporate methanol in hot water bath under stream of nitrogen. Dissolve aflatoxin in 2.0 mL HPLC mobile phase, and seal vial.

(e) *Methanol–NaCl solution.*—Add 100 mL reagent grade methanol to 100 mL 5% NaCl in distilled water. Store in stoppered Erlenmeyer flask.

(f) *Sodium sulfate.*—Anhydrous, granular (MCB SX760).

Extraction

Extraction and cleanup steps are derived from ref. 4.

Weigh 25 g spice (ground to pass U.S. No. 20 mesh sieve) into 500 mL Erlenmeyer flask, add 12.5 mL saturated NaCl solution and 125 mL dichloromethane (CH_2Cl_2), secure flask stopper with masking tape, and shake 30 min on mechanical shaker. Gravity-filter 10 mL of this solution through Whatman No. 4 paper. Recycle the 10 mL filtrate through the filter and then filter 50 mL of solution and collect filtrate in graduated cylinder. Transfer filtrate to 250 mL glass-stopper Erylenmeyer flask. (This recycling pretreats filter paper and produces cleaner extract.) Add boiling chips to flask and evaporate extract to near dryness in hot water bath.

Cleanup

Add 100 mL methanol–NaCl solution and 50 mL hexane to Erlenmeyer flask containing residue, secure stopper with masking tape, and shake 10 min on mechanical shaker. Transfer mixture to 250 mL separatory funnel and let stand 5 min. Drain lower layer into another 250 mL separatory funnel. Add 50 mL CCl_4 to this second funnel and shake vigorously ca 1 min. Discard lower layer. Repeat partition with 25 ml CCl_4. Add 50 mL CH_2Cl_2 to retained layer and shake ca 1 min. Drain CH_2Cl_2 layer into 125 mL Erlenmeyer flask and repeat extraction with another 25 mL CH_2Cl_2. Combine CH_2Cl_2 extracts, add boiling chips to flask, and evaporate to near dryness in hot water bath.

Column Chromatography

Place ball of glass wool in bottom of chromatographic column, and add ca 1 cm anhydrous Na_2SO_4. Add CH_2Cl_2 until column is one-third full. Weigh 2.0 g 70–230 mesh silica gel into 30 mL beaker. Slurry gel with ca 15 mL CH_2Cl_2 and completely transfer to column. After gel settles, tap in ca 2 cm layer of anhydrous Na_2SO_4. Drain CH_2Cl_2 to top of Na_2SO_4 and inspect gel layer for air bubbles. If any are present, remove by filling reservoir halfway with CH_2Cl_2 and forcing bubbles out with compressed air. Drain CH_2Cl_2 to top of Na_2SO_4.

Dissolve extract in 5 mL CH_2Cl_2 and add extract solution to column. Wash sides of flask with two 5 mL portions of CH_2Cl_2 and add

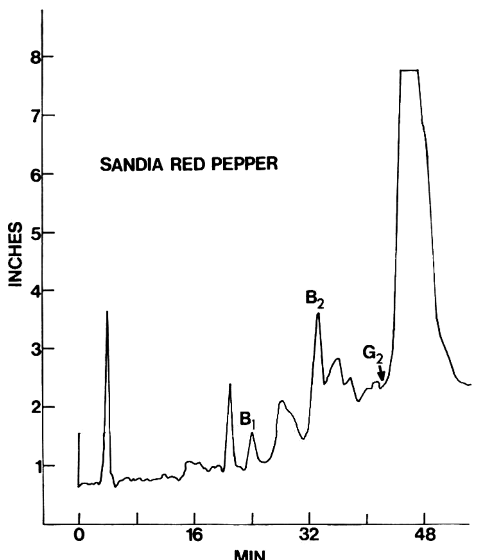

Figure 1. Chromatogram of 0.9 g Sandia red pepper containing 0.2 μg aflatoxins B_1 and B_2/kg.

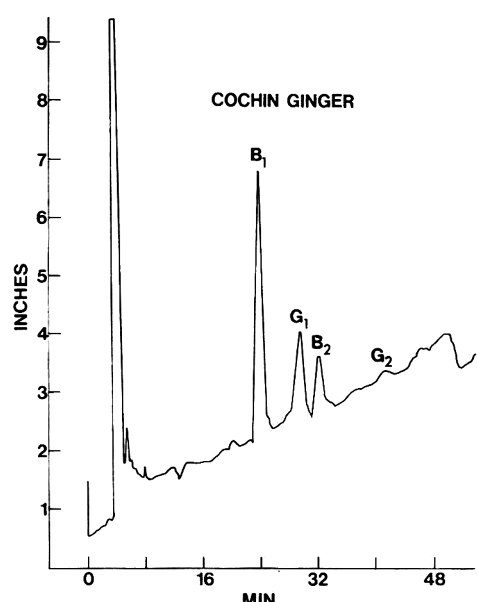

Figure 2. Chromatogram of 0.8 g Cochin ginger containing 6.5 μg aflatoxin B_1/kg, 2.5 μg G_1/kg, 0.2 μg B_2/kg, and 0.2 μg G_2/kg. The sample was reinjected at attenuation 5 to obtain a higher G_2 peak.

washings to column. Elute sequentially at maximum flow rate with 40 mL CH_2Cl_2, 40 mL benzene–acetic acid (9 + 1), 40 mL hexane, and 40 mL ether; discard eluates. After each addition of solvent or extract, drain column until liquid level meets top of packed bed. Elute aflatoxins with 130 mL HPLC mobile phase. When analyzing black and white pepper, this entire column chromatographic step must be performed twice on the extract so that severe negative interfering peaks and late-eluting peaks are removed. Collect eluate in 250 mL Erlenmeyer flask until flow stops. Add boiling chips to flask and evaporate to near dryness in hot water bath.

High Pressure Liquid Chromatography

Stabilize instrument at flow rate of 0.75 mL/min; pressure at column head should be ca 600 psi. Operate detector with lamp at high setting, gain at low, and attenuation at 10–20. Maximize fluorescence signal from detector by injecting B_1 standard solution into analytical column under operating conditions and stopping flow when B_1 is at its maximum in packed flowcell. Adjust light source and flowcell position to maximize signal.

Quantitatively transfer extract with CH_2Cl_2 from Erlenmeyer flask to concentrator tube. Completely remove solvent with heat and nitrogen stream, and dissolve extract in 0.5 mL mobile phase. Inject 15–30 μL aflatoxin standard solution (which equals 5, 10, 1, and 2 μg/kg of B_1, G_1, B_2, and G_2, respectively, in 10 g of spice sample). Record retention time and either integrator areas or peak heights. In same manner, inject 15–30 μL sample extract and record chromatogram. Calculate amount of each aflatoxin (μg/kg) as described by Pons and Franz (14).

Confirmation of Aflatoxin Identity

Prepare TLC plate and solvents as described by Suzuki et al. (1). Redissolve final extract in $CHCl_3$ and spot aliquot and develop according to ref. 1 except eliminate methanol–phosphoric acid spraying. After 2-dimensional development, spray plate with 25% aqueous H_2SO_4, and view under longwave ultraviolet light. Aflatoxins B_1 and B_2 turn from blue to bright yellow fluorescence; G_1 and G_2 turn from bluish green to bluish yellow fluorescence. Color change is indicative but not absolute proof of the presence of aflatoxin (12).

Results and Discussion

Resolution and Detection

The specified HPLC mobile phase and instrument parameters elute all 4 aflatoxins in about 45 min. This is over 4 times longer than observed by Pons and Franz (10) using almost

Table 1. Recovery (μg/kg) of 4 aflatoxins from ginger by HPLC[a]

Aflatoxin	Added, μg/kg	Rec., range	Mean and SD[b]	Mean rec., %
B_1	25	13.5–19.1	16.1 (2.3)	64
	5	2.9–3.8	3.4 (0.4)	68
B_2	5	3.2–4.1	3.7 (0.4)	74
	1	0.78–0.82	0.78 (0.05)	78
G_1	50	38.0–43.4	41.0 (2.6)	82
	10	7.9–10.3	9.1 (1.2)	91
G_2	10	7.2–9.6	8.2 (0.1)	82
	2	1.4–1.9	1.7 (0.2)	85

[a] See text for description of operating conditions.
[b] Four determinations at each level.

Table 3. Recovery (μg/kg) of 4 aflatoxins from white pepper by HPLC[a]

Aflatoxin	Added, μg/kg	Rec., range	Mean and SD[b]	Mean rec., %
B_1	25	15.6–18.2	16.9 (1.2)	68
	5	3.1–3.9	3.6 (0.3)	72
B_2	5	2.9–4.1	3.4 (0.6)	68
	1	0.63–0.83	0.73 (0.08)	73
G_1	50	35.1–40.5	37.9 (2.7)	76
	10	6.6–8.4	7.5 (0.1)	75
G_2	10	6.4–8.5	7.3 (1.1)	73
	2	1.4–1.6	1.5 (0.1)	75

[a] See text for description of operating conditions.
[b] Four determinations at each level.

exactly the same column (4.0 mm id instead of our 3.9 mm id; both columns 30 cm long) from the same manufacturer, and with 1.5% added ethanol instead of the 0.5% used in this work. When we tried using 1.5% ethanol at 1.0 mL/min, we obtained very poor resolution between the G_1 and B_2 peaks. We also noted that contrary to the observation of Pons and Franz, the positions of the G_1 and B_2 peaks were reversed so that the elution order was B_1, G_1, B_2, G_2. This observation was confirmed by injecting solutions of individual aflatoxin standards. This order was observed previously by Seitz (15) who used a similar 10 μm silica gel column with a CH_2Cl_2 mobile phase containing 0.6–0.8% methanol. Aflatoxin sensitivity was reduced in our case due to broader peaks but was more than adequate to detect 0.3 μg/kg levels of B_1 in the spice extracts. We attribute the difference in column performance to variations in the manufacturing process as noted in refs. 9 and 11.

Extraction and Cleanup

It was discovered early in our study that additional cleanup steps were necessary for both black and white pepper due both to severe negative peaks in the aflatoxin region and to large and very late-eluting interferences. Replacing the silica gel-packed flowcell with an unpacked one removed the negative peaks entirely. This suggests that the background signal of the detector depends heavily on the "fluorescence" radiation of the silica gel itself in the flowcell and that any substance that absorbs part of this radiation reduces this background signal and produces a negative peak. Removing the negative interference by using an unpacked cell was not possible with this solvent system. As expected, the B_1 and B_2 sensitivities were drastically reduced (10).

After performing the column chromatographic step on a white or black pepper extract, using the $CHCl_3$–acetone (4 + 1) elution solvent specified by Stoloff and Trucksess (4) for ginger, we noticed a great deal of dark, almost irreversibly adsorbed material covering the entire silica gel column. We believed that this dark material severely overloaded the column and thus did not allow separation of the early-eluting aflatoxins

Table 2. Recovery (μg/kg) of 4 aflatoxins from red pepper by HPLC[a]

Aflatoxin	Added, μg/kg	Rec., range	Mean and SD[b]	Mean rec., %
B_1	25	18.2–22.1	20.6 (1.7)	82
	5	3.8–5.0	4.6 (0.5)	92
B_2	5	3.9–4.7	4.3 (0.3)	86
	1	0.76–0.98	0.88 (0.1)	88
G_1	50	41.0–49.5	43.8 (3.9)	88
	10	6.1–10.6	9.1 (2.0)	91
G_2	10	8.3–10.0	8.8 (0.8)	88
	2	1.4–2.0	1.8 (0.5)	90

[a] See text for description of operating conditions.
[b] Four determinations at each level.

Table 4. Summary of spices analyzed for aflatoxins

Spice	No. analyzed	No. varieties	No. samples contg aflatoxins
Black pepper	12	4[a]	0
White pepper	7	1[b]	1
Red pepper	15	7[c]	8
Ginger	5	2[d]	3
Nutmeg	3	1[e]	0

[a] Lampong (3), Malabar (5), Sarawak (2), and Sri Lanka (2).
[b] Muntok.
[c] Sandia (5), Bahamian (2), Cheng-tu (4), Wang-tu (1), Yuman (1), Jalapeno (1), and Pakistan (1).
[d] Cochin (3) and Jamaican (2).
[e] East Indian (3).

Table 5. Aflatoxin content of positive red pepper, white pepper, and ginger samples

Spice	Variety	Aflatoxin concn, µg/kg			
		B_1	G_1	B_2	G_2
Red pepper	Cheng-tu	32.9	28.4	1.5	1.1
	Cheng-tu[a]	13.4	4.4	0.5	—
	Cheng-tu	3.6	0.7	0.1	—
	Cheng-tu[a]	8.6	2.8	0.2	—
	Sandia	0.2	—	0.2	—
	Sandia	0.7	—	0.1	—
	Sandia	0.2	—	0.2	—
	Pakistan	4.9	—	0.2	0.5
Ginger	Cochin	6.5	2.5	0.2	0.2
	Cochin	1.4	—	—	—
	Cochin	4.2	—	0.2	—
White pepper	Muntok	0.3	—	—	—

[a] Same lot as sample on line immediately above.

from the later-eluting interferences. A weaker elution solvent was tried, and the HPLC solvent was selected because it was noticed that precipitation occurred in the extraction solutions dissolved in the HPLC solvent stored in the freezer. Analysis of these precipitated solutions showed a marked reduction in the size of the late-eluting interferences. Thus, the fact that the interferences had a limited solubility in this solvent seemed to point to its use in eluting the aflatoxins from the 2 g silica gel cleanup column. Also, silica gel that was not treated with 1% water was used to provide a more active adsorptive surface.

A single pass through the cleanup column employing the above variations was not satisfactory. Running the extract through a second new silica gel column provided complete removal of late-eluting interferences and the negative peaks. This technique of passing the extract through 2 fresh silica gel columns was repeated on a new pepper extract, using the original $CHCl_3$-acetone elution solvent. The negative peaks in the aflatoxin region were eliminated, but the late-eluting ones were still very prominent.

It was discovered later that 2 exposures to a silica gel surface combined with the exposure to the acetic acid in the benzene–acetic acid wash was required to entirely remove the negative interferences. Evidently, these substances are relatively unstable in an acidic environment.

To avoid having to put the black and white pepper extracts through 2 full size cleanup columns, we studied the use of a minicolumn such as the widely used Sep-Pak columns. We discovered, however, that the minicolumns did not retain a sufficient amount of the late-eluters so that after first going through a minicolumn and then a full size column, the late interferences were reduced but were still present. Gradient elution was not available to us but could be used in this case to hasten the elution of the interferences. A secondary solvent with a greater absolute ethanol content would be applicable.

HPLC Chromatograms

In the analysis of ginger, red pepper, and nutmeg, nutmeg produced the cleanest chromatogram, being totally free of any interferences. In the Jalapeno and in some Sandia red pepper samples, large positive peaks eluted near the aflatoxin G_2 region and made positive identification difficult. An HPLC chromatogram of a Sandia red pepper in which this interference occurs is shown in Figure 1. In ginger, a small negative peak eluting right between the G_1 and B_2 peaks was troublesome only when large aliquots of the sample extract were injected when determining aflatoxin in the 0.1–1.0 µg/kg range. The chromatogram of a sample of Cochin ginger contaminated with all 4 aflatoxins is shown in Figure 2.

Recovery of Aflatoxins

Tables 1, 2, and 3 show the results obtained for the recovery of aflatoxins added at 2 different levels to aflatoxin-free ginger, red pepper, and white pepper, respectively. A formal study was not performed on nutmeg, but recoveries were about 80% for all aflatoxins in this spice. The 5, 10, 1, and 2 µg/kg levels of B_1, G_1, B_2, and G_2, respectively, produced similar peak heights for all aflatoxins. This relative sensitivity among aflatoxins is somewhat different from that obtained by Pons (11).

Table 4 lists the number and type of spices analyzed in this study, and the number of samples with positive aflatoxin results. Table 5 lists the concentrations of the individual aflatoxins found in the positive samples. As expected, the incidence of aflatoxin contamination was highest in red pepper and ginger. The white pepper sample containing 0.3 μg B_1/kg is the first positive result reported for aflatoxin in this spice.

Acknowledgment

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Citrus Artifact Interference in Aflatoxin M_1 Determination in Milk

RALPH L. PRICE, KAREN V. JORGENSEN, and MICHAEL BILLOTTE[1]
University of Arizona, Department of Nutrition and Food Science, Tucson, AZ 85721

Dried citrus waste was fed to dairy cows, their milk was extracted, and aflatoxin M_1 was quantitated by using both high pressure liquid chromatography (HPLC) and thin layer chromatography (TLC). Results indicate that a compound from the citrus waste, which is excreted into the milk, interferes with the HPLC determination of aflatoxin M_1 in milk and causes a false positive test. This interference can be overcome by using TLC with proper selection of developing solvents.

In May 1980, milk from a commercial dairy in the Phoenix, Arizona area was tested for aflatoxin M_1 by United Dairymen of Arizona and was found to contain up to 0.2 μg M_1/L. The milk was extracted as described by Stubblefield (1), and aflatoxin M_1 was quantitated by high pressure liquid chromatography with fluorescence detection. Because the dairy ration had been formulated from supposedly noncontaminated ingredients, duplicate feed and milk samples were tested by this laboratory for verification of previous results. The milk samples were extracted by the procedure of Stubblefield (1), and were visually quantitated by 2-dimensional thin layer chromatography (TLC) (2). The feed samples, mixed grain and dried citrus waste, were analyzed by the AOAC official first action method for aflatoxin B_1, **26.032-26.036** (3), as is routinely done in this laboratory. The milk samples were aflatoxin-free; however, we observed a new, blue fluorescing compound appearing at a slightly higher R_f value. Confirmation by the trifluoroacetic acid (TFA) technique (2) verified that this compound was not M_1. However, when the compound was quantitated as M_1, the levels were equivalent to the M_1 levels found by United Dairymen of Arizona. The feed samples were also aflatoxin-free. Citrus extracts contained numerous fluorescing compounds; confirmation with TFA showed that none of the compounds was aflatoxin.

Because citrus contains many flavonoids and coumarin derivatives (4) and some of these interfere with B_1 analysis in mixed feeds (5), we suspected that one of these compounds is excreted into the milk as is aflatoxin. This study was designed to determine if feeding dried citrus waste to lactating cows causes a false positive M_1 test or interference in M_1 detection when certain analytical techniques are used. This was accomplished by conducting a feeding study, and then sampling the milk and determining M_1 and the unknown compound (artifact). Once the artifact was found, the R_f values were compared to determine which solvent system(s) gave the best separation of the 2 compounds on both 1- and 2-dimensional TLC. The extracts containing the artifact were also analyzed by HPLC in an attempt to overcome this interference.

Experimental

Feeding Trial

Cottonseed and dried citrus waste were obtained from a commercial dairy in the Phoenix, Arizona area. Mixed grain and alfalfa were supplied by the University of Arizona dairy farm. Before being fed, the cottonseed was analyzed for aflatoxin by AOAC official first action methods **26.A10-26.A14, 26.A15** (5), and dried citrus waste was analyzed by the method of Jain and Hatch for citrus-containing feeds (6).

Four Holstein cows were chosen from the University of Arizona dairy herd. Each cow was kept in a separate pen to control feeding. One cow was maintained as a control and received normal dairy rations consisting of 24 lb commercial daily concentrate (mixed grain) and 20 lb alfalfa cubes/day. The second cow received normal dairy rations consisting of 24 lb commercial dairy concentrate (mixed grain) and 20 lb alfalfa cubes/day. The second cow received the normal ration plus both the cottonseed and dried citrus waste.

Milk samples were taken at each milking for a 10-day period, beginning once before feeding the dried citrus waste and cottonseed and continuing throughout the trial. Two 300 mL milk samples from each milking were analyzed within 15 h of sampling.

Milk Analysis

All milk samples were masked before analysis. The milk was analyzed by the method of Stubblefield (1) and aflatoxin M_1 was confirmed as

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[1] United Dairymen of Arizona, PO Box 26877, Tempe, AZ 85282.

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Table 1. R_f values obtained by 1-dimensional TLC of aflatoxin M_1 and artifact in milk, with several solvent systems

Developing solvent	M_1	Artifact
$CHCl_3$-acetone-isopropanol (87 + 10 + 3)	0.32	0.59
Ether-methanol-water (95 + 4 + 1)	0.44	0.63
$CHCl_3$-acetone (9 + 1)	0.67	NS[a]
Toluene-ethyl acetate-formic acid (6 + 3 + 1)	0.20	NS
$CHCl_3$-acetone-isopropanol (85 + 10 + 5)	0.62	NS
Ether-hexane-methanol-water (85 + 10 + 4)	0.27	0.70
$CHCl_3$-acetone-isopropanol (85 + 10 + 7)	0.76	NS

[a] NS = no separation.

described by Trucksess (2). Thin layer plates, 20 × 10 cm, 0.5 mm thick (Adsorbosil-1, Applied Science, State College, PA 16801), were developed for 8 cm in an unequilibrated tank with ether-methanol-water (95 + 4 + 1). The plates were dried in the dark at room temperature, and then were developed for 8 cm in a second direction with $CHCl_3$-acetone-isopropanol (87 + 10 + 3). Aflatoxin M_1 was visually quantitated by comparing a known amount of M_1 standard with the quantity extracted from the milk. The artifact was quantitated as M_1.

HPLC analysis was performed on 20 μL aliquots of the sample extracts. The HPLC system included a Waters HPLC modular unit, μC_{18} reverse phase column, M60^0A pump, Wisp 710A automatic injector, 420E fluorescence detector

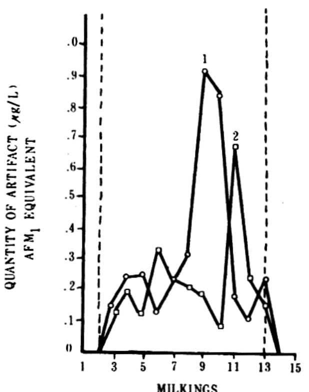

Figure 1. Levels of artifact found in milk from cows fed dried citrus waste: (1) milk from cow fed dried citrus and normal ration; and (2) milk from cow fed dried citrus, cottonseed, and normal ration.

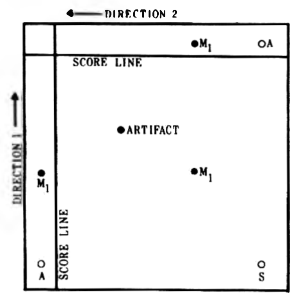

Figure 2. Two-dimensional thin layer chromatogram of aflatoxin M_1 and citrus artifact.

(365–400 nm), 440 UV detector (365 nm), and a data module integrator. The mobile phase was water-methanol-acetonitrile (59 + 22 + 19) at a flow rate of 1.0 mL/min.

R_f Value Comparision

Two 50 mL milk samples containing the artifact were spiked with aflatoxin M_1 standard and analyzed according to Stubblefield (1). A 20 μL aliquot of the sample was spotted along with a 5 μL aliquot of M_1 standard, and developed by 1-dimensional TLC with the solvent systems shown in Table 1. The R_f values were measured and calculated as described by Touchstone and Dobbins (7). The distance from the origin to the solvent front was kept constant at 10 cm. This procedure was repeated with 2-dimensional TLC with a constant 8 cm solvent front.

Results and Discussion

Studies by Polan et al. (8) and Patterson et al. (9) showed that M_1 accumulates in the milk and increases continuously as long as aflatoxin is fed. They also showed that M_1 was detected in the milk 24 h after initial feeding and up to 72 h after aflatoxin was removed from the diet. Results from our study (Figure 1) show that the artifact appeared in the milk within 12 h after the citrus

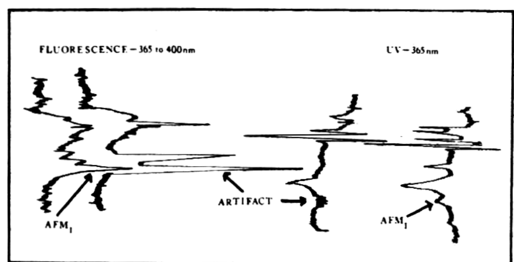

Figure 3. HPLC chromatograms of aflatoxin M_1 and citrus artifact. Retention time: M_1 6.86 min and artifact 7.18 min.

Table 2. Effect of eluant on column chromatographic separation of aflatoxin M_1 and artifact [a]

Eluant [b]	M_1	Artifact
Hexane [c]	–	–
Ethyl ether	–	–
$CHCl_3$-acetone (9 + 1)	+	+
$CHCl_3$-acetone (1 + 4)	+	+
$CHCl_3$-methanol (97 + 3)	+	+
Methanol	+	+
Benzene-acetic acid-methanol (90 + 5 + 5)	–	–
Acetonitrile-ether-hexane (4 + 1 + 5)	–	–
Benzene-hexane (1 + 1)	–	–

[a] (+) = eluted from column; (–) = no effect.
[b] Eluant volume 25 mL except as noted.
[c] Eluant volume 50 mL.

waste was first fed and continued as long as it was fed. Although these results show that the artifact was excreted into the milk, levels did not increase continuously as does M_1 but fluctuated considerably. They also show that the artifact was not detected in the first milk samples taken after the citrus waste was removed from the diet (12 h). These results corroborate the previous findings of United Dairymen of Arizona that the levels of the supposed aflatoxin M_1 found in the milk were inconsistent. No unusual fluorescent compounds were detected in milk from cows not fed dried citrus waste.

The results of comparison of R_f values are shown in Table 1. The solvent systems that gave the best separation for 1-dimensional TLC were $CHCl_3$–acetone–isopropanol (87 + 10 + 3); ether–methanol–water (95 + 4 + 1); and ether–hexane–methanol–water (85 + 10 + 4 + 1). In the other solvent systems, streaking made it difficult to distinguish the artifact from M_1.

Figure 2 shows the results obtained for 2-dimensional TLC. The solvent systems consisted of ether–methanol–water (95 + 4 + 1) in the first direction and $CHCl_3$–acetone–isopropanol (87 + 10 + 3) in the second direction. These solvent systems clearly separated the 2 compounds. The results from both the 1- and 2-dimensional TLC comparisons showed an obvious separation of aflatoxin M_1 and the artifact. Because it takes less time, our laboratory prefers to use 1-dimensional TLC.

Figure 3 is a comparison of the HPLC chromatograms for 2 milk extracts, one containing the artifact and one containing M_1. The fluorescence detector showed both the M_1 and the artifact peaking at the same position. The UV detector gave a positive response for M_1 but gave a negative response for the artifact. We attempted to determine the presence of the artifact by establishing a UV/fluorescence response factor ratio but this was effective only if the artifact appeared alone and in large quantities. If the milk contained both the artifact and M_1, HPLC indicated a much higher level of M_1 than was actually present. Derivatization with TFA by the method of Beebe and Takahashi (10) did not produce enough shift for differentiation of the 2 compounds by HPLC.

Because of the problems encountered with HPLC quantitation when either the artifact or both were present, attempts were made to separate the 2 by varying conditions during the column chromatographic step of the Stubblefield method (1). Different eluants were used as additional washes (Table 2), and eluates were examined by TLC to determine which compound(s) was removed. The moisture content of the silica gel was also varied (0, 2, 3, 4, and 5% water). None of the modifications was effective.

This experience again emphasizes the need for confirmation of analytical procedures for aflatoxins by methods other than cochromatography. This will aid in avoiding the imposition of penalties on producers of noncontaminated commodities.

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DRUG RESIDUES IN ANIMAL TISSUES

Comparison of Three Methods for Determination of Sulfamethazine in Swine Tissues: Collaborative Study

ANTHONY J. MALANOSKI, CHARLIE J. BARNES,[1] and THOMAS FAZIO[1]
U.S. Department of Agriculture, Food Safety and Quality Service, Division of Chemistry, Washington, DC 20750

Collaborators: D. Andrzejewski; R. G. Burkepile; D. Goodspeed; J. D. Jackson; J. Jones; A. Manuel; J. Matusik; W. J. Morris; R. K. Munns; G. J. Nandrea; J. Nandrea; M. Poeppel; J. Shafer; R. Simpson; S. Stout; F. Suhre; R. R. Wise

An interlaboratory study of 3 methods for the determination of sulfamethazine in swine muscle and liver tissue was conducted. A gas chromatographic (GC), a combined gas chromatographic-mass spectrometric (GC/MS), and a colorimetric method were evaluated. Four fortified and 2 incurred levels of sulfamethazine ranging from 0.00 to 0.45 ppm were studied. The study revealed that the GC/MS procedure provides the most reliable data and that the GC method provides acceptable data with the incorporation of appropriate controls. The recoveries for the GC/MS method typically ranged from 90 to 100% for swine liver and muscle. The GC and GC/MS methods have been adopted official first action.

Sulfonamides have been approved for use in food-producing animals for approximately 40 years. Tishler's (colorimetric) modification (1) of the Bratton-Marshall procedure (2) in 1968 was a great step forward in the ability to determine sulfonamide residues in animal tissues. Since 1968 the procedures or modifications of this system, such as the Fellig-Westheimer method (3), have been most widely used in sulfonamide analyses.

For some time, the Food and Drug Administration (FDA) and the Food Safety and Quality Service (FSQS) have been concerned about deficiencies in methodology based on the Bratton-Marshall reaction for determining sulfonamides. Background interferences from control tissues are frequently 50% or more of the 0.1 ppm tolerance. Recoveries are sometimes low and variable. The Bratton-Marshall types of methods generally do not distinguish one sulfonamide from another or from other compounds that react in a similar manner.

In 1978, high rates of violative sulfonamide residues were occurring in swine liver. As a result, major aspects of the sulfonamide monitoring program were scrutinized, including the analytical methodology, and FSQS and FDA evaluated and standardized their application of the Tishler procedure. Because of the problems and limitation of the Tishler procedure, a decision was made to develop a suitable replacement. The outcome of these investigations was the development of (a) a combined gas chromatography-mass spectrometry (GC/MS) method for sulfamethazine by FSQS (4) and (b) a slightly modified version of the Manuel-Steller (GC) (5) sulfonamide method by FDA. The GC and GC/MS procedures, together with Option A of the Tishler procedure, were subjected to interlaboratory study.

Samples

Animals for this study were received as gifts from the Science Education Administration, U.S. Department of Agriculture. These animals were fed and dosed by Lowell Frobiak of the Non-Ruminant Nutrition Laboratory, Agriculture Research Center, Beltsville, MD.

Tissues from the slaughtered animals were screened by a TLC-fluorescence procedure before the start of the study. In this pre-assay screening procedure, tissue was blended with acetone and the resulting mixture was centrifuged; the acetone layer was filtered, 5N HCl was added, and the solution was buffered to adjust the pH to 6.2. The acid solution of the residues was extracted with methylene chloride. A concentrated methylene chloride extract of the residues was subjected to TLC. Sulfamethazine and other sulfonamides present were made visible by a Bratton-Marshall type of color reaction.

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[1] Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

The recommendation was approved by the General Referee and Committee G and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 65, March issue (1982) for detailed reports.

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In brief, the protocol called for the analyses of blind replicates of 3 levels of fortified samples, blank tissue samples, and 2 levels of incurred residue samples, each in both swine liver and muscle. (Incurred residues are the parent drug and metabolites that remain in the animal as a result of animal dosing.) Coded weighed samples and matching vials for fortifying these tissues were distributed to each analyst along with a schedule for doing the analyses. Each sample was fortified with 1 mL of the contents of a designated vial just before sample analyses. Vials for blanks and incurred samples contained methanol but no sulfamethazine. Samples were generally analyzed at the rate of 4 per day in a specific random order for 3 days per week. This provided an opportunity to make up for contingencies.

Tishler A Colorimetric Method (1)

Extract 50 g tissue 3 times with 100 mL chloroform–acetone (1 + 1). Filter and rotary-evaporate at 50°C; dissolve residue in hexane–acetone (50 + 1). Extract 4 times with 1N HCl. Filter 1N HCl extract and adjust to pH 14. Wash basic extract with $CHCl_3$, adjust to pH 1.0, and diazotize with sodium nitrite. Destroy excess $NaNO_3$ with $NH_4OSO_2NH_2$, and filter through fine fritted funnel. For color, add *N*-(1-naphthyl)ethylenediamine.HCl. Read complex at 545 nm.

Gas Chromatographic-Mass Spectrometric Method (4)

Add ^{13}C-sulfamethazine to 50 g tissue, and proceed as in colorimetric method above, through "Wash basic extract with $CHCl_3$." Buffer acid extract and adjust to pH 5.6. Extract residues into CH_2Cl_2 and evaporate. Dissolve in MeOH and methylate with diazomethane. By GC/EI/MS, monitor m/z 227, 228, 233, 234, 92, and the absence of m/z 74. Quantitate by use of the ratio of 227 to 233.

Manuel-Steller GC Method (5)

Extract 15 g tissue with 150 mL chloroform–acetone (1 + 1), filter, and collect 100 mL. Add 10 mL 1N HCl, and rotary-evaporate at 32°C to remove extraction solvent. Add 50 mL hexane, shake, and remove 1N HCl layer. Extract twice more with 5 mL 1N HCl. Buffer 1N HCl, adjust to pH 5.6, and extract with CH_2Cl_2. Rotary-evaporate, dissolve residue in acetone, and methylate with diazomethane. Quantitate by electron capture GLC.

Results and Discussion

The 3 methods under study have similar extraction and cleanup steps and differ principally in the procedures used for detection and quantitation. The GC/MS method uses a ^{13}C-sulfamethazine internal standard, the extraction and cleanup of the Tishler-A, and quantitation by combined GC/MS. The GC method uses many

Table 1. GC recovery of sulfamethazine from swine muscle (ppm)

		Sample description		Participating laboratory				
Animal	No.	Treatment	Pre-test assay[a]	1	2	3	4	5
5703	5	blanks (fortified	0.00 ppm by Manuel-	0.00	0.00	0.00	0.00	0.01
	12	at zero sulfa-	Steller plus TLC/	0.00	0.00	0.00	0.00	0.00
	17	methazine level)	fluorescence screen	—	—	—	—	—
5703	6	fortified 0.05 ppm		0.05	—	0.05	0.06	0.07
	11		none	0.05	0.03	0.05	0.01	0.03
	14			—	0.07	—	—	—
5703	3	fortified 0.11 ppm		0.11	0.06	0.09	0.11	0.08
	10		none	0.10	0.07	0.10	0.05	0.07
	16			—	—	—	—	—
5703	8	fortified 0.22 ppm		0.21	0.22	—	0.24	0.18
	9		none	0.21	—	0.22	0.25	0.22
	13			—	—	0.23	0.20	—
5704	2	incurred	0.22 ppm by TLC/	0.18	0.23	0.21	0.25	0.13
	4		fluorescence screen	0.24	—	0.25	0.26	0.23
	18			—	0.48	—	—	—
5706	1	incurred	0.45 ppm by TLC/	0.53	0.45	loss	0.16	0.25
	7		fluorescence screen	0.40	discard	0.53	0.52	0.36
	15			—	—	—	—	—

[a] Performed on blanks and incurred samples only.

Table 2. GC recovery of sulfamethazine from swine liver (ppm)

	Sample description			Participating laboratory				
Animal	No.	Treatment	Pre-test assay[a]	1	2	3	4	5
A	2	blanks (fortified	0.00 ppm by Manuel-	0.01	0.02	0.00	0.00	0.00
+	12	at zero sulfa-	Steller plus TLC/	—	0.03	0.01	0.00	0.00
B	16	methazine level)	fluorescence screen	0.02	—	—	—	—
A	1	fortified 0.06 ppm		0.05	0.12	0.06	0.07	0.06
+	4		none	0.05	0.12	0.05	0.06	—
B	13			—	—	—	—	0.05
A	5	fortified 0.12 ppm		0.11	0.13	0.12	0.12	0.09
+	10		none	0.09	0.14	0.12	0.13	0.10
B	17			—	—	—	—	—
A	8	fortified 0.18 ppm		0.14	0.18	0.14	0.13	0.10
+	11		none	0.15	0.19	0.20	0.21	—
B	18			—	—	—	—	0.13
5704 F	3	incurred	0.05 ppm by TLC/	0.05	0.07	0.05	0.04	—
	9		fluorescence screen	0.05	0.07	0.04	0.06	0.04
	14			—	—	—	—	0.03
5709 F	6	incurred	0.08 ppm by TLC/	0.05	0.07	0.08	0.08	—
	7		fluorescence screen	0.04	0.09	0.08	0.06	0.06
	15			—	—	—	—	0.05

[a] Performed on blanks and incurred samples only.

of the same solvents as the Tishler-A, but the number of extractions differ, and quantitation is achieved by electron capture gas chromatography rather than by the Bratton-Marshall colorimetric procedure. Quantitation in both the GC/MS and GC methods uses the methyl derivative of sulfamethazine. The methods have been published (4, 5). Full details will be given in "Changes in Methods," in the March 1982 issue of the AOAC Journal.

The data obtained by the participating laboratories are shown in Tables 1–6. Included in these tables are the identity of the animals from which the tissue samples were taken, the sample and reserve sample numbers, and the results of the pre-test and test assays.

Four different groups independently provided us with statistical evaluations of the recovery data. These statistical treatments differed primarily in the corrections applied to the data generated by the Tishler-A and GC methods. One statistician adjusted the results by the GC and Tishler-A for background and recovery. In another treatment, laboratory performance standards were developed and some laboratories were excluded for failure to meet these stan-

Table 3. GC/MS recovery of sulfamethazine from swine muscle (ppm)

	Sample description			Participating laboratory				
Animal	No.	Treatment	Pre-test assay[a]	1	2	3	4	5
5703	4	blanks (fortified	0.00 ppm by TLC/	0.00	0.00	0.03	0.01	0.00
	10	at zero sulfa-	fluorescence screen	0.01	0.00	0.02	0.00	0.00
	15	methazine level)	plus Manuel-Steller	—	—	—	—	—
5703	3	fortified 0.05 ppm		0.06	0.05	0.06	0.05	0.05
	7		none	0.06	0.05	0.08	0.05	0.05
	13			—	—	—	—	—
5703	11	fortified 0.11 ppm		0.11	0.10	0.14	0.10	0.11
	12		none	0.11	0.12	0.12	0.10	0.10
	16			—	—	—	—	—
5703	5	fortified 0.22 ppm		0.21	0.27	0.25	0.20	0.20
	6		none	0.22	0.22	—	0.20	0.20
	17			—	—	0.24	—	—
5704	8	incurred	0.22 ppm by TLC/	0.22	0.24	0.24	0.21	0.22
	9		fluorescence screen	0.22	0.24	0.25	0.20	0.24
	14			—	—	—	—	—
5706	1	incurred	0.45 ppm by TLC/	0.45	0.45	0.46	0.41	0.46
	2		fluorescence screen	0.44	0.34	0.46	0.37	0.46
	18			—	—	—	—	—

[a] Performed on blanks and incurred samples only.

Table 4. GC/MS recovery of sulfamethazine from swine liver (ppm)

Animal	No.	Sample description: Treatment	Sample description: Pre-test assay[a]	Participating laboratory: 1	2	3	4	5
1	4	blanks (fortified	0.00 ppm by TLC/	0.00	0.00	0.01	0.00	0.00
+	11	at zero sulfa-	fluorescence screen	0.00	0.00	0.02	0.00	0.01
3	17	methazine level)		—	—	—	—	—
1	1	fortified 0.06 ppm		0.06	0.05	0.06	0.06	0.05
+	8		none	0.06	0.06	0.06	0.06	0.06
3	18			—	—	—	—	—
1	5	fortified 0.12 ppm		0.12	0.13	0.13	0.11	0.13
+	10		none	0.12	0.11	0.14	0.11	0.10
3	15			—	—	—	—	—
1	2	fortified 0.18 ppm		0.18	0.18	0.18	0.16	0.18
+	7		none	0.18	0.16	0.19	0.17	0.18
3	16			—	—	—	—	—
5701 F	6	incurred	0.14 ppm by TLC/	0.14	0.18	0.15	0.13	0.14
	12		fluorescence screen	0.14	0.15	0.16	0.13	0.15
	14			—	—	—	—	—
5702 F	3	incurred	0.07 ppm by TLC/	0.06	0.06	0.07	0.06	0.05
	9		fluorescence screen	0.07	0.07	0.06	0.05	0.06
	13			—	—	—	—	—

[a] Performed on blanks and incurred samples only.

dards. In 2 of the treatments, various tests were used to eliminate the outlier values. One statistician applied a recently published concept (6) to determine the lowest limit of reliable measurement (Lm). Lm is defined as that level which gives a response above the expected blank value greater than or equal to 0.75 times the spread of the 99% confidence limits on a single assay response measured parallel to the observed assay response axis. For a full explanation and a diagram of this concept, the reference should be consulted.

Although independently evaluated by 4 different groups, the final conclusions drawn were essentially the same. The major discrepancies in the data were those for muscle (*see* values 33 and 40, A for GLC and B for Tishler-A in Table 7). The method providing the most reliable data was the GC/MS method; the GC was second and Tishler-A was last. A summation of the data derived from the 4 statistical reports (A, B, C, D) is shown in Table 7.

Considering the statistical data and the operational characteristics of each method, our as-

Table 5. Tishler-A recovery of sulfamethazine from swine muscle (ppm)

Animal	No.	Sample description: Treatment	Sample description: Pre-test assay[a]	Participating laboratory[b]: 1	2	4	5
5703	3	blanks (fortified	0.00 ppm by TLC/	0.03	0.01	0.03	0.03
	12	at zero sulfa-	fluorescence screen	0.01	—	0.01	0.02
	18	methazine level)	plus Manuel-Steller	—	0.02	—	—
5703	1	fortified 0.05 ppm		0.04	0.05	0.03	0.04
	7		none	0.04	0.04	0.14	0.05
	13			—	—	—	—
5703	4	fortified 0.11 ppm		0.05	0.08	0.07	0.07
	11		none	0.04	—	0.15	0.07
	17			—	0.06	—	—
5703	5	fortified 0.22 ppm		0.16	0.13	0.14	0.16
	10		none	0.18	—	0.17	0.17
	16			—	0.14	—	—
5704	2	incurred	0.22 ppm by TLC/	0.16	0.17	0.15	0.17
	9		fluorescence screen	0.20	0.16	0.07	0.18
	15			—	—	—	—
5706	6	incurred	0.45 ppm by TLC/	0.42	0.28	0.30	0.33
	8		fluorescence screen	0.30	0.27	0.20	0.32
	14			—	—	—	—

[a] Performed on blanks and incurred samples only.
[b] Laboratory 3 did not have time to complete the analyses.

Table 6. Tishler-A recovery of sulfamethazine from swime liver (ppm)

		Sample description		Participating laboratory[b]			
Animal	No.	Treatment	Pre-test assay[a]	1	2	4	5
B_1, B_4	9	blanks (fortified	0.04, 0.07, 0.08, 0.04	0.11	0.07	0.07	0.09
B_5, B_6	10	at zero sulfa-	ppm by Tishler-A	0.12	0.07	0.06	0.13
	16	methazine level)		—	—	—	—
B_1, B_4	4	fortified 0.06 ppm		0.12	0.09	0.08	0.14
B_5, B_6	5		none	0.14	0.10	0.09	0.12
B_1, B_4	2	fortified 0.12 ppm		0.16	0.16	0.12	0.16
B_5, B_6	12		none	0.17	0.15	0.11	0.17
	18			—	—	—	—
B_1, B_4	7	fortified 0.18 ppm		0.17	0.16	0.24	0.19
B_5, B_6	11		none	0.19	0.18	0.17	0.21
5705 M	1	incurred	0.06 ppm by TLC/	0.12	0.08	0.11	0.13
	6		fluorescence screen	0.14	0.08	0.09	0.19
	15			—	—	—	—
5702 M	3	incurred	0.13 ppm by TLC/	—	0.25	0.26	0.30
	8		fluorescence screen	0.27	0.23	0.19	0.33
	13			0.28	—	—	—

[a] Performed on blanks and incurred samples only.
[b] Laboratory 3 did not have time to complete the analyses.

sessments of the 3 methods are as follows:

The GC/MS has a degree of specificity, precision, and accuracy and is relatively unaffected by tissue background interferences. The high precision and accuracy of the method are probably due to the use of the internal standard. Potential limitations of the GC/MS method are that it requires a ^{13}C-labeled sulfamethazine for use as the internal standard, a mass spectrometer, and a residue chemist trained in the use of the GC/MS instrument.

With respect to limit of reliable measurement, specificity, and freedom from background interferences the GC method is not as good as the GC/MS method but is better than the Tishler-A method. Data generated in this study show that with respect to repeatability and reproducibility this method was less satisfactory than the Tishler-A method. However, the Lm in both swine liver and muscle is lower for the GC than for the Tishler-A method. The higher Lm for the Tishler-A method possibly reflects the problem

Table 7. Summation of statistical evaluations

	Liver			Muscle		
Evaluation	CV[a]	CV[b]	Lm	CV[a]	CV[b]	Lm
GC/MS						
A	9	9	0.034	8	12	0.058
B	8	9		7	13	
C		9			12	
D	9	10		10	13	
GLC						
A	15	22	0.096	33	33	0.074
B	14	16		15	19	
C		20			20	
D	13	22		21	21	
Tishler-A						
A	11	19	0.174	13	16	0.100
B	25	27		38	40	
C		17			14	
D	8	17		13	14	

[a] Coefficient of variation of repeatability (within laboratories).
[b] Coefficient of variation of reproducibility (between laboratories).

with higher background interferences in blank tissue for that method.

There are several possible reasons why the GC method did not perform better in this study. Some of the variable results might be due to steps in the procedure that need optimization.

Because of time constraints, it was decided not to have each analyst run a daily sample of known sulfamethazine content as a check on the performance of the method. Such assay controls would have provided the analyst with daily feedback on incidence of serious problems with the Tishler-A and the GC methods in this study. The Tishler-A modification of the Bratton-Marshall procedure, although not specific as a result of background interferences, remains useful as a screening procedure for sulfonamides in all edible animal tissue.

Conclusion and Recommendation

Results of this study indicate that the GC/MS procedure provides the most reliable data and should be used for determining sulfamethazine in edible swine tissue. The Manuel-Steller GC method will also provide acceptable data when used with the appropriate controls. The procedures will be extended to determination of other sulfonamides in other species of food-producing animals.

It is recommended that the GC/MS and the Manuel-Steller GC methods be adopted official first action.

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For protocol design: Raymond B. Ashworth, Harry M. Marks, John McNeal, Robert Epstein, and Richard Ellis, FSQS, Washington, DC; John W. Howard and William Horwitz, FDA, Washington, DC.

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MEAT AND MEAT PRODUCTS

Multicomponent Analysis of Meat Products

OLE-CHRISTIAN BJARNØ
A/S N. Foss Electric, 69, Slangerupgade, Hillerød DK-3400, Denmark

A new method for rapid multicomponent analysis of meat products is reported. The sample is homogenized, and then dispersed by treatment with an alkaline aqueous solution combined with mechanical subdivision. Constituents are determined by infrared transmission spectrophotometry in the fundamental IR absorption bands. It is possible to measure protein, fat, and carbohydrate in about 5 min with an accuracy and precision comparable to that of well established standard methods, and no expensive or dangerous chemicals are used.

There is a great demand for quantitatively determining fat and protein in foodstuffs and animal feeds and their raw materials. Until a few years ago, the principal method for protein determination was the Kjeldahl method, in a modified automated form (1). Color-binding (2) and the biuret reaction (3) had gained a certain acceptance, but only for determining dissolved proteins, for example, protein in milk and serum (4).

Likewise, the principal method for fat determination has been extraction with organic solvents, and determination, on the clear extract, of a physical property indicative of fat content, e.g., dielectric constant, refractive index, or density, as with the Foss-Let automated instrumentation (5). For special purposes, such as fat determination in milk, measurements by light scattering have been widely used (6).

Both of the principal methods (Kjeldahl and extraction) suffer from the disadvantages that they are difficult to perform, use toxic chemicals, and yield toxic waste fluids. Both biuret and color-binding suffer from the disadvantage that the calibration strongly depends on the product to be determined.

It was a technological advance when Goulden (7) showed that it was possible to quantitatively measure protein and fat in milk by infrared (IR) spectrophotometry. Until then, this had been considered impossible because of the strong water bands; when Goulden published his work, spectrophotometry technology had been developed so far that it was possible to compensate efficiently for interferences. Goulden's work formed the basis of several IR transmission instruments for routine analysis of milk (8-10).

The use of infrared technology for solid products has also been introduced (11, 12). The determinative step in this case is limited to the measurement of reflected light from the surface of a finely ground sample. Also, it is necessary to operate in the near IR range to obtain sufficient energy, which necessitates the use of the less sharp overtones of the fundamental IR bands for protein and fat. This fact, in connection with the high sensitivity of the technique to variations in the sample surface, resulted in a less than satisfactory accuracy and precision (12), but the method is relatively fast and does not use toxic chemicals.

The near IR reflection method has also been applied to meat products (13, 14). However, there exists a great demand for a *precise* and *accurate* method for determining fat, protein, carbohydrate, water, and ash in meat products. The evaluation of such a method is discussed in this report.

Experimental

The infrared analysis of milk products is a well established technique which normally works with an accuracy about 1.5% relative on all 3 parameters: fat, protein, and lactose according to the AOAC interim official first action method (**16.079** (15); official first action (16)). To apply this technique and its high accuracy to analyzing meat, it is necessary to convert the meat sample to a milk-like emulsion.

Meat will give stable emulsions under appropriate mechanical treatment. In addition, alkaline solutions markedly enhance solubilization of meat protein (17). To convert the meat sample to a milk-like emulsion, we mixed 11 g meat with 100 mL 0.1M sodium hydroxide. The sample size of 11 g corresponds to about 5 g mean dry matter. The maximum content of dry matter that can be emulsified and give stable emulsions is about 10 g. To accommodate normal variations and to ensure a margin of safety, 11 g is adequate. The mechanical treatment is performed by using

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a Foss-Let reactor (5) in which the mixture is subjected to treatment by a reciprocating cylindrical hammer in a closed chamber. To establish a more stable emulsion ca 0.1% of a mixture of different emulsifiers is added to the alkaline reaction solution. No chemical reactions take place. However, during the mechanical subdivision, carbohydrates are solubilized in the water phase; fat is melted and emulsified. Other laboratory homogenizers have been investigated, including the Ultra Turrax, but the former treatment is more efficient.

The sample is once more homogenized by a built-in homogenizer and then transferred to the cell for IR measurements. The absorption wavelengths used are the carbon-oxygen double bond (5.73 μm) for fat, the nitrogen-hydrogen bond (6.5 μm) for protein, and the carbon-oxygen bond attached to the hydroxyl groups (9.5 μm) for carbohydrates, all wavelengths at which water absorbs. This water interference can be eliminated by measuring all components at 2 different wavelengths: one where the compound in question has an absorption maximum, and another adjacent wavelength, where the compound does not absorb energy. The necessary wavelengths are selected by means of dielectric interference filters placed on a filter wheel. The rest of the optical system is almost identical to other spectrophotometers.

The signal from each channel, which is the ratio between the signals from the sample and reference filters, is corrected with ca 5% of the signals from the other 2 channels. These corrections are necessary because of (*1*) Spectral influences: Components also absorb in other channels. (*2*) Water displacement effects: When a component in one of the 2 other channels is varied, it gives a variation in the water background. Because of incomplete compensation by the reference wavelength, this variation generates variation in the actual measuring channel. (*3*) Density changes: A variation in any component will be reflected in the other two because density of the emulsion is changed.

Standard methods used in meat analysis differ from IR measurement because the former measures a weight-fraction and the latter measures mole-fraction. Therefore, results will be influenced by density variations. Only when the molecular weight is constant from sample to sample for the component in question will the IR measurement give a constant accuracy vs the standard method.

Calculations on pig fat show that only minor variation in mean molecular weight would be expected, although a considerable variation exists in the fatty acid composition.

The mean molecular weight is also a source of error in Kjeldahl determination of protein because of the varying Kjeldahl factor (the conversion factor between percent nitrogen and percent protein). Normally, 6.25 is used as the conversion factor but the following factors have been calculated based on biochemical data (18):

Muscle protein	5.94
Collagen (connective tissue)	4.94
Hemoglobin (blood)	5.65

Samples with high and varying content of collagen must have the Kjeldahl protein determinatin corrected. For meat proteins, very little variation other than collagen is present.

The molweight of carbohydrates varies, but is generally compensated by variation in number of carbon-oxygen bonds. For example, glucose has a molweight of 180.17 and 6 carbon-oxygen bonds, and sucrose has a molweight of 342.70 and 11 carbon-oxygen bonds. Thus, for a certain weight-fraction of sucrose, the same weight-fraction of glucose will measure about 3.5% higher relative. This difference will be reduced in actual products where the variation between monosaccharides and disaccharides is more limited.

Results

First, a spectrum of a meat emulsion was recorded to investigate the absorption frequencies of the components. An emulsion was established by reacting 12 and 6 g beef in 100 mL 0.5M NaOH in a Foss-Let reactor for 4 min. The emulsion was transferred to a 30 μm cell and a difference spectrum was recorded with a Perkin-Elmer infrared spectrophotometer 580 with 0.5M NaOH in the reference cell. Figure 1 shows the fat absorption at 1740 cm^{-1} and protein absorption at 1560 cm^{-1}, which are the frequencies used for infrared milk measurements. Furthermore, it is seen that the ratio between sample wavelength and reference wavelength roughly estimates the content of the specific components.

Next, a Milko-Scan 104, a standard instrument for milk measurements, was used (9). Thirty sample mixtures were prepared so that the most common meat constituents were randomized; only small intercorrelations of the constituents should be present. The standard methods applied in analyzing the mixtures were Kjel-Foss automated for protein determination (AOAC **24.037**) and Foss-Let (AOAC **24.008**) for fat de-

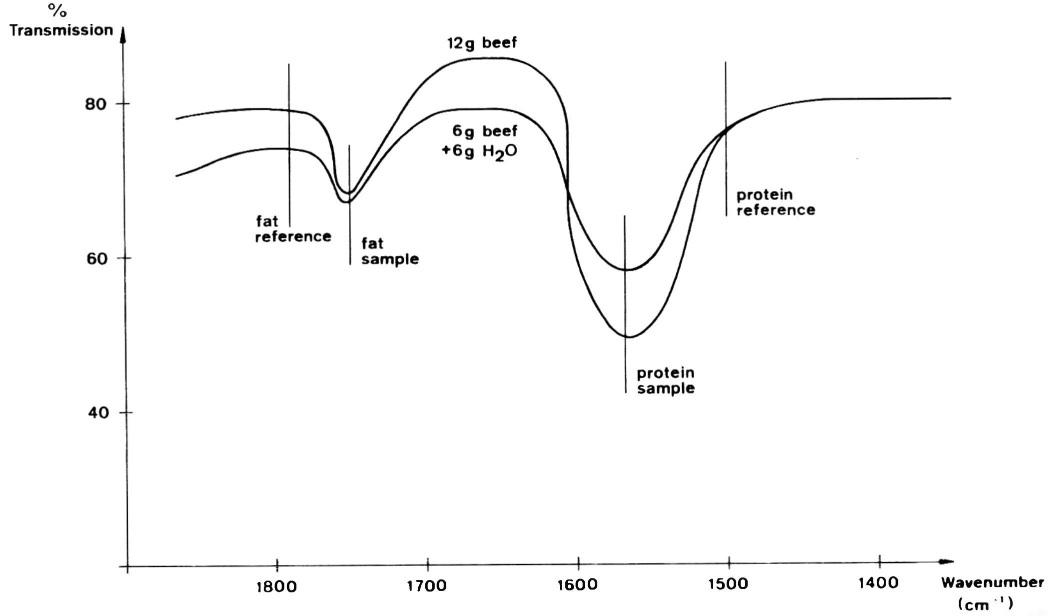

Figure 1. Infrared spectrum of emulsions of 12 and 6 g meat samples, showing fat absorption at 1740 cm^{-1} and protein absorption at 1560 cm^{-1}.

termination. The carbohydrate content was estimated by assuming that nonprocessed meat material does not contain significant carbohydrate and that potato flour consists of only protein, carbohydrate, and water; the latter was assayed by oven drying at 105°C to constant weight. Mixtures and results are presented in Table 1. Statistics (19) are summarized in Table 2.

A variety of samples was chosen, 4 pork meats, 4 beef meats, 5 liver sausages, and 3 cooked sausages, so that the parameters were uniformly distributed between 8.7 and 50.5% fat and 10.18 and 18.28% protein (maximum content of connective tissue protein is 25%). The sample was finely homogenized on a double knife chopper with 2 mm holes (Möhle Boy FW 70N). Samples were weighed for duplicate determinations by the standard methods (Kjel-Foss automatic and Foss-Let) as well as duplicate determinations by IR spectrophotometry (Super-Scan, an instrument from N. Foss Electric A/S, Denmark, dedicated to transmission measurements on meat products). All samples were subjected to alkaline treatment before introduction to the systems, as given in the procedure.

The results from the instruments were handled by singular regression analysis vs the standard results, and subjected to analysis of variance. Results are reported in Table 3.

In the final experiment, 3 g dried analytical grade carbohydrate was mixed with 8 g homogenized beef, treated according to the standard procedure, and measured on 2 IR instruments (Table 4).

One instrument response closely resembles the theoretical content (27.3%); the second instrument varies more. This variance may be due to filter tolerances, but needs further investigation.

Discussion

Statistical results compare with those obtained by the standard methods in which both repeatability and between-laboratory standard deviation is about 0.3%. For another multicomponent method, near IR reflection, very little is reported, but repeatability and reproducibility seem to be about 2–3 times higher (13, 14).

The coefficients in the regression equations are very much like those obtained by milk measurements; the latter are theoretically based on and verified for several hundred instruments for several years of operation. This emphasizes the theoretically sound basis of IR transmission measurements for meat products.

Neck tendon protein was introduced to determine interferences. Meat proteins other than muscle proteins have a different peptide bond molweight ratio as well as a different Kjeldahl

Table 1. Comparison of standard method and IR results for components in meat mixtures

	Constituent, %								Found, %					
									Fat		Protein		Carbohydrate	
Sample No.	Beef	Pork	Ox fat	Pork fat	Water	Potato flour	NaCl	Neck tendon from ox	Std	IR	Std	IR	Std	IR
1	95	0	0	0	0	2	3	0	4.0	4.0	21.8	22.1	2.0	2.0
2	93	0	5	0	0	1	1	0	8.3	8.6	21.5	22.0	1.0	1.3
3	90	0	0	0	0	5	5	0	3.8	3.9	20.7	20.9	5.0	5.2
4	85	0	5	0	0	3	2	5	8.8	9.2	21.3	21.4	3.0	3.6
5	76	0	17	0	3	0	4	0	18.1	18.1	17.9	18.4	0.0	0.3
6	75	0	10	0	0	3	2	10	13.6	13.8	20.9	20.3	3.0	3.0
7	70	0	5	0	11	6	3	5	8.1	8.2	17.9	17.7	6.0	5.9
8	65	0	15	0	15	0	0	5	16.7	17.0	17.0	16.9	0.0	0.0
9	60	0	8	0	28	4	0	0	9.6	10.0	14.0	14.4	4.0	4.6
10	40	0	16	0	43	1	0	0	15.8	15.7	9.6	10.2	1.0	1.3
11	0	95	0	0	0	2	3	0	3.4	3.3	19.4	18.9	2.0	1.8
12	0	93	0	5	0	1	1	0	7.5	8.2	19.2	18.8	1.0	0.9
13	0	90	0	0	0	5	5	0	3.2	3.0	18.4	18.2	5.0	5.0
14	0	85	0	5	0	3	2	5	8.1	8.1	19.2	18.8	3.0	3.0
15	0	76	0	17	3	0	4	0	17.1	17.2	16.3	16.1	9.9	10.1
16	0	75	0	10	0	3	2	10	12.7	12.5	19.1	18.5	3.0	2.3
17	0	70	0	5	11	6	3	5	7.5	7.3	16.2	15.6	6.0	5.7
18	0	65	0	15	15	0	0	5	15.8	15.8	15.6	15.0	0.0	0.0
19	0	60	0	8	28	4	0	0	8.9	9.0	12.6	12.6	4.0	4.0
20	0	40	0	16	43	1	0	0	15.0	15.1	8.9	9.0	1.0	1.0
21	25	70	0	0	0	2	3	0	3.5	3.3	20.0	19.8	2.0	2.1
22	70	23	5	0	0	1	1	0	8.2	7.8	20.9	21.4	1.0	1.1
23	20	70	0	0	0	5	5	0	3.3	3.1	18.9	18.8	5.0	5.1
24	65	20	0	5	0	3	2	5	8.5	8.6	20.9	22.2	3.0	2.9
25	15	61	17	0	3	0	4	0	17.8	17.2	16.4	16.5	0.0	0.0
26	60	15	0	10	0	3	2	10	13.1	12.4	20.6	20.5	3.0	2.6
27	10	60	5	0	11	6	3	5	7.8	7.7	16.4	16.3	6.0	5.8
28	55	10	0	15	15	0	0	5	16.2	16.1	17.0	16.1	0.0	0.0
29	5	55	8	0	28	4	0	0	9.2	9.2	12.6	13.0	4.0	4.0
30	35	5	0	16	43	1	0	0	15.2	15.2	9.8	10.4	1.0	1.0
Corr. coeff.									0.998		0.991		0.991	

Regression equations:

$$F = 1.15 \times F^* + 0.05 \times P^* + 0.09 \times C^* - 1.13$$
$$P = 0.93 \times P^* + 0.09 \times F^* + 0.05 \times C^* + 0.39$$
$$C = 0.64 \times C^* + 0.08 \times P^* + 0.06 \times F^* - 0.39$$

factor, but at this level no systematic interference is seen. In the same manner, the variations in salt content do not interfere.

This report appears to be the first for assaying carbohydrate by a rapid instrumental method. The experiment with different carbohydrates shows that the variation in performance is somewhat higher than what could be expected from theoretical considerations, and these problems need further investigation.

Table 2. Statistical results for IR analysis of 30 mixtures of meat components

Statistic	Fat, %	Protein, %	Carbohydrate, %
Repeatability SD, S_O [a]	0.35	0.25	0.30
Between method SD, S_M [b]	0.13	0.45	0.19

[a] Repeatability of method.
[b] Variation of difference between means of standard method and IR method.

Conclusion

The new, rapid instrumental method for determination of components in meat seems to have a precision superior to near IR reflexion measurements and at the same level as standard

Table 3. Statistical analysis of results obtained on a group of 17 different meat products

Statistic	Fat, %	Protein, %
Repeatability SD, S_O	0.30	0.20
Between instruments SD, S_I [a]	0.20	0.16
Between method SD, S_M	0.48	0.22
Reproducibility SD, $S_O + S_I$	0.58	0.26
Accuracy SD, $S_I + S_M$	0.52	0.27

[a] Variation of sample results between instruments.

Table 4. Instrument performance for carbohydrates

Carbohydrate	Instrument 1, %	Instrument 2, %
D-Glucose	27.80	28.70
D-Galactose	27.17	25.93
Lactose	27.00	27.05

methods. This new method will be further investigated through collaborative studies.

Acknowledgment

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Interaction Between Sample Preparation Techniques and Colorimetric Reagents in Nitrite Analysis in Meat

JAY B. FOX, TATIANA E. ZELL, and AARON E. WASSERMAN
U.S. Department of Agriculture, Agricultural Research, Eastern Regional Research Center, Philadelphia, PA 19118

The amount of nitrite measured in model and meat systems was a function of the interactions of the chloride and ascorbate concentrations with the method of sample preparation and the combination of Griess reagents used for colorimetric determination. Ascorbate caused loss of nitrite in the samples when heated and interfered in the Griess reaction, increasing the concentration of pigment formed from any given concentration of nitrite if sulfanilic acid and *N*-(1-naphthyl)-ethylenediamine were used, and decreasing the amount if sulfanilamide and 1-naphthylamine were used. The interference was eliminated by both the AOAC procedure and mercuric chloride addition, although the former were less effective at higher ascorbate concentrations. Chloride increased the amount of pigment formed from a given amount of nitrite with sulfanilic acid but had no effect on the amount of sulfanilamide pigment.

Although sample preparation for the analysis of nitrite in meat products varies with respect to degree of dilution, temperature of heating, alkalinization, or addition of various precipitating agents, the most common method of analysis is the spectrophotometric measurement of a diazo pigment formed from some combination of Griess reagents (1-8). The amount of pigment formed is affected by the presence of ascorbate (1, 2) and chloride (3). Ascorbate reduces the formation of carcinogenic nitrosamines in cured meat products (9) but the addition of large amounts of ascorbate interferes in the Griess reaction. Low yields of pigment, therefore, could be due either to a real loss of nitrite in the meats or to the ascorbate interference. Until now there has been no way of distinguishing these 2 effects, but it has been reported recently that ascorbate will either enhance or decrease the amount of pigment formed, depending on the combination of Griess reagents used (10).

The purpose of this study was to determine if this differential effect can be used to detect residual ascorbate in cured meats and to determine the effectiveness of sample preparation procedures on removal of ascorbate. We used the specific combinations of sulfanilic acid (SAA) with *N*-(1-naphthyl)-ethylenediamine (NED) and sulfanilamide (SAN) with 1-naphthylamine (1-NA), because these 2 combinations showed the greatest differences between the amount of pigment produced from any given amount of nitrite in the presence of ascorbate. For brevity, we shall refer to the first combination as SAA, and the second as SAN.

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Experimental

Model System

Four model stock solutions were prepared in ionic strength $\Gamma/2 = 0.03$ acetate buffer, pH 5.5, each containing 2 mM nitrite and either 0, 3, 6, or 12 mM ascorbate. Half of each solution was made 1M in NaCl by addition of the appropriate amount of solid salt. All solutions were analyzed for nitrite, and then heated 1 h at 70°C in air. We chose pH 5.5 as the lower end of the range of meat pH values, to ensure an appreciable nitrite loss.

Meat System

A slurry was prepared by blending pork semitendinosus muscle with an equal volume (w/v) of 6 mM sodium nitrite. Aliquots of the slurry were combined with half their volumes of solutions containing 0, 9, 18, or 36 mM sodium ascorbate, respectively. Each of these slurries was then divided into 3 aliquots and NaCl was added so that samples at every level of sodium ascorbate contained 0, 0.5, or 1.0M NaCl. Half (10 mL) of each of the 12 samples was placed in a controlled water bath 1 h at 70°C. All of the 24 (12 raw and 12 cooked) samples were analyzed for nitrite as soon as prepared. The pH was 5.62.

Nitrite Analyses

Direct analysis.—Aliquots of the slurries were centrifuged 20 min at 48 200 g. The clear supernates were carefully removed with a syringe fitted with a 28 gauge needle. One-tenth mL was added to 1.0 mL of each appropriate Griess reagent solution in a 10 mL volumetric flask and diluted to volume. Turbidities of the pigment solutions were determined at 600 nm but were not of practical significance.

Mercuric chloride addition.— A saturated mer-

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Table 1. Effects of NaCl, ascorbate, sample preparation methods, heat, and Griess reagent on measured nitrite ($mM\ NO_2^-$) in model systems [a] (pH 5.5; $[NO_2^-]$ initial = 2.0 mM)

		Unheated						Heated 70°C, 1 h					
		Direct		$HgCl_2$		AOAC		Direct		$HgCl_2$		AOAC	
M NaCl	mM Asc.	SAA	SAN	SAA	SAN	SAA	SAN	SAA	SAN	SAA	SAN	SAA	SAN
0	0	2.09	1.91	2.11	1.96	2.04	2.00	1.92	1.93	2.00	2.02	1.84	2.00
0	3	2.32	1.09	2.07	1.98	1.94	1.93	2.04	1.15	1.75	1.74	1.73	1.76
0	6	2.11	0.75	2.05	1.93	1.96	1.90	1.98	0.55	1.62	1.62	1.68	1.63
0	12	1.64	0.44	2.05	1.96	1.85	1.78	1.33	0.33	1.47	1.48	1.36	1.37
1	0	2.43	1.96	2.25	2.02	1.99	2.00	2.41	1.96	2.30	2.00	2.00	2.02
1	3	2.61	1.13	2.20	2.00	1.99	1.97	2.47	1.06	2.00	1.78	1.70	1.87
1	6	2.23	0.73	2.15	1.96	1.91	1.87	2.04	0.42	1.83	1.69	1.90	1.75
1	12	1.70	0.45	2.08	1.93	1.77	1.86	1.27	0.30	1.57	1.42	1.24	1.33

[a] SAA = the reagent combination of sulfanilic acid and *N*-(1-naphthyl)ethylenediamine. SAN = reagent combination of sulfanilamide and 1-naphthylamine.

curic chloride solution, 0.2 mL, was added to 2.0 mL slurry and mixed. The sample was then treated as described for direct analysis.

*AOAC method, **24.041-24.042*** (4).—One mL samples were measured by positive displacement from a calibrated syringe into 100 mL volumetric flasks and diluted to ca 80 mL with water. Solutions were heated 2 h at 80°C, diluted to volume, and filtered through Whatman No. 2V paper. (Filter paper has been shown to be contaminated with nitrite (5) which interferes in the accuracy of the analysis. Sen and Donaldson (6) recommended discarding the first 20 mL filtrate but we found it necessary to wash the filters with 300-400 mL distilled water before washings were color-free with Griess reagents.) To 8 mL filtrate in 10 mL volumetric flask, 1 mL Griess reagent was added and the solution was diluted to volume. These solutions were then analyzed for nitrite.

Griess reagent combinations.—Two combinations used were 1.0 mM sulfanilic acid with 0.2 mM *N*-(1-naphthyl)-ethylenediamine (SAA), and 1.0 mM sulfanilamide with 0.2 mM 1-naphthylamine (SAN) prepared in 15% acetic acid. OSHA has classified 1-naphthylamine as a toxic and hazardous substance (CFR 29:1910.1004, 1979). The pigment concentrations in standard and sample solutions were determined from their absorbances. The absorptivities for standard solutions of sodium nitrite with NaCl or ascorbate added were a_{mM} = 22.0 at 540 nm for SAA and 42.5 at 525 nm for SAN.

Results

Model System

The results are shown in Table 1. In the absence of NaCl and ascorbate (first row), the concentration of nitrite measured was not affected by heat, sample preparation, or Griess reagent combination. The coefficient of variation of results for samples in the first row of Table 1 was 4%, which agrees well with the CV of 3% determined independently by replicate analyses of samples prepared by the 3 methods.

Two effects of ascorbate were observed on the nitrite measurement by direct analysis. The SAA reagent combination (column 3) increased apparent nitrite concentration with a maximum at 3 mM ascorbate, followed by a decrease at higher concentration. With the SAN reagents (column 4), a continuous decrease in apparent nitrite concentration with increasing levels of ascorbate was noted.

Both mercuric chloride and AOAC treatments eliminated the effects of ascorbate on the concentration of nitrite measured by both SAA and SAN reagent combinations in the unheated samples, although the AOAC procedure was not completely effective at the highest ascorbate concentration. When samples were heated, there was a loss of nitrite in the samples containing ascorbate, but the presence of residual ascorbate was indicated by the difference in nitrite concentrations measured by the 2 reagents in the direct reading samples. Again, AOAC and $HgCl_2$ treatments were effective in eliminating the ascorbate effect on pigment formation. There was also a chloride effect on the nitrite concentration measured by the 2 reagent combinations. Except in one set, the SAA values were always higher than the SAN values in the samples containing chloride, whether from NaCl or $HgCl_2$. Hildrum (3) has shown that chloride enhances pigment formation with sulfanilic acid, but in separate tests we found that chloride does

Table 2. Effects of NaCl, ascorbate, sample preparation methods, heat, and Griess reagents on measured nitrite ($mM\ NO_2^-$) in meat slurry [a] (pH 5.6; $[NO_2^-]$ initial = 2.1 mM (145 ppm))

		Raw						Cooked					
		Direct		$HgCl_2$		AOAC		Direct		$HgCl_2$		AOAC	
M NaCl	mM Asc.	SAA [a]	SAN	SAA	SAN	SAA	SAN	SAA	SAN	SAA	SAN	SAA	SAN
0	0	2.23	2.15	1.79	1.70	2.07	1.90	1.85	1.87	1.64	1.37	2.01	1.91
0	3	2.79	1.64	1.76	1.65	2.21	1.83	2.09	1.38	1.36	1.27	2.00	1.76
0	6	2.63	1.27	1.72	1.63	2.62	1.61	1.98	1.01	1.33	1.20	2.04	1.54
0	12	2.29	0.81	1.77	1.67	2.70	1.28	1.58	0.66	1.22	1.13	1.91	1.16
0.5	0	2.33	2.40	2.29	2.07	1.82	1.73	2.09	1.79	2.02	1.76	1.98	1.91
0.5	3	2.72	1.61	2.07	1.97	1.86	1.63	1.84	1.19	1.60	1.39	1.45	1.43
0.5	6	2.72	1.24	2.00	1.90	2.14	1.56	1.58	0.87	1.45	1.28	1.45	1.24
0.5	12	2.33	0.81	2.00	1.93	2.37	1.26	1.35	0.53	1.31	1.18	1.45	0.93
1	0	2.45	1.91	2.16	1.97	2.01	1.85	2.20	1.83	2.03	1.71	1.84	1.76
1	3	2.42	1.62	2.08	1.91	2.08	1.78	1.98	1.17	1.71	1.30	1.54	1.37
1	6	2.44	1.20	2.03	1.84	2.50	1.29	1.49	0.71	1.36	1.07	1.30	1.02
1	12	1.84	0.81	2.18	1.87	2.20	1.27	0.93	0.39	1.03	0.86	1.09	0.73

[a] SAA = reagent combination of sulfanilic acid and *N*-(1-naphthyl)ethylenediamine. SAN = sulfanilamide and 1-naphthylamine.

not affect the amount of pigment formed from sulfanilamide. The difference is therefore due to the differential effect of chloride on the nitrosation of the 2 reagents.

Meat Slurry

Heat caused a loss of nitrite in all but 3 of the meat slurry samples (Table 2). The loss in the samples with no added reductant (first row) was about 10% (significant at $P = 0.02$ from paired variate t-test) and was due to reaction with endogenous compounds in meat. An examination of all data, especially from the ascorbate-containing samples, shows that the measured nitrite was not uniform for any one of the factors studied, but was the result of interactions with the other experimental conditions. An analysis of variance (Table 3) showed that the variations could be accounted for by 4 three-factor interactions. Since the mean values for the 3-way combinations most clearly illustrate the more salient features of the corresponding 3-factor interactions, they are shown graphically in Figures 1–4.

Heat Processing × Salt × Ascorbate.—The mean values ($n = 6$) for the first combination in Table 3 are shown in Figure 1. Measured nitrite decreases in both raw and cooked samples with both salt and ascorbate. Because the raw samples were analyzed for nitrite immediately after being prepared, there was little time for nitrite to react with tissue components or added ascorbate, and the observed regression is due to ascorbate interference in the Griess reaction. Although chloride has no effect on the measured concentration of nitrite in the raw samples, the loss of nitrite in the cooked samples due to ascorbate was enhanced by increasing chloride concentration. Assuming nitrite loss on heating to be

Table 3. Significant 3-factor interactions from analysis of variance of data of Table 2

Interaction	*F* ratio	Significance, *p*
Processing × salt × ascorbate	3.42	0.033
Processing × preparation × reagent	20.3	0.0001
Processing × reagent × ascorbate	10.9	0.0010
Prepartion × reagent × ascorbate	22.9	0.0001

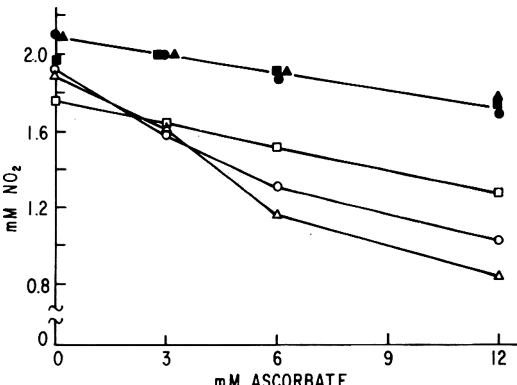

Figure 1. Mean values ($n = 6$) illustrating 3-factor interaction of heat, salt, and ascorbate concentration effects on measured nitrite in pork slurry. □, 0 salt; ○, 0.5M NaCl; △, 1.0M NaCl. Solid symbols, unheated; open symbols, heated.

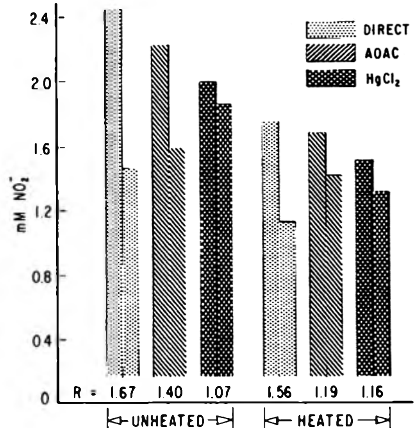

Figure 2. Mean values (n = 12) illustrating 3-factor interaction of heat, preparation method, and reagent on measured nitrite. First bar in pair SAA, second SAN. Figure below reagent pair is ratio of SAA to SAN values.

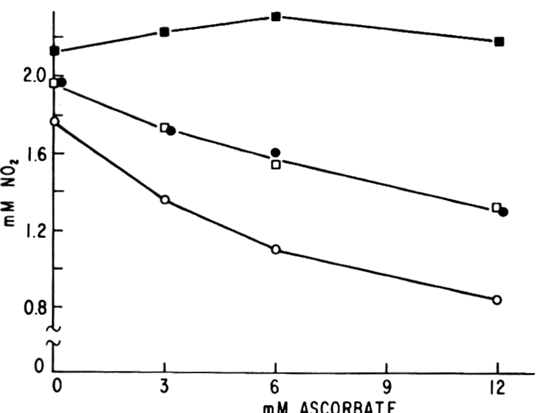

Figure 3. Mean values (n = 9) illustrating 3-factor interaction of heat, reagent, and ascorbate concentration on measured nitrite. □, SAA; ○, SAN. Solid symbols, unheated; open symbols, heated.

related to the reactivity of the nitrosating species, this chloride enhancement may be attributed to the formation of nitrosylchloride (NOCl), a more reactive nitrosating species than N_2O_3, the species formed from nitrite alone (11). On the basis of the observation that loss of nitrite was not proportional to chloride level in the model system, it may be attributable to NOCl being more reactive than N_2O_3 with meat tissue components.

Heat Processing × Preparation × Reagent.—The difference in measured nitrite by the 2 colorimetric reagents (Table 2) was a function of both processing and method of preparation (second interaction, Table 3). The mean values (n = 12) are plotted in Figure 2. In addition to the decrease in measured nitrite after heating, the difference between the 2 colorimetric reagents was less in the cooked than in the raw samples for both the direct and AOAC preparation methods. As was observed in the model system, the reagent difference is due to residual ascorbate, which was only partially destroyed in the meat slurries by the heating process. The difference was minimal in the $HgCl_2$ samples because ascorbate was removed by precipitation with mercuric ion, but the chloride effect was still present.

Heat Processing × Reagent × Ascorbate.—Table 2 shows that, although the nitrite concentrations in the unheated samples measured by SAA were relatively constant, there was a regression with ascorbate in the heated/SAA samples and in all the SAN samples. This 3-factor interaction was significant (P = 0.0001); the mean values (n = 9) are shown in Figure 3. In the unheated samples the ascorbate enhancement of pigment formation from the SAA reagent is observed, as well as the decrease in the pigment formed from the SAN reagent. In the heated samples, the SAA values were uniformly higher than the SAN values, due to both the ascorbate and chloride enhancement of SAA pigment formation, but there was a significant regression of measured nitrite with ascorbate in the SAA samples. The coincidence of the SAA/heated and SAN/unheated data is happenstance and has no significance.

Preparation × Reagent × Ascorbate.—The fourth 3-factor interaction in Table 3 is the most relevant to the intent of this study in that it shows the effect of the sample preparation techniques on the ascorbate interaction with the 2 colorimetric reagents. The mean values (n = 6) for this interaction are shown in Figure 4. The regressions of measured nitrite with ascorbate concentration are not linear because nitrite loss is a function of the square root of the ascorbate concentration (12, 13). The differential effects of both chloride and ascorbate on pigment production are observed in the curves of the direct and AOAC procedures, but only the differential chloride effect on pigment production from the 2 reagent combinations in the $HgCl_2$ procedure. The initial values differ because of the chloride effect, but the direct and AOAC curves diverge with increasing ascorbate, indicating residual ascorbate. Because the AOAC procedure was effective in removing ascorbate in the model systems, it is evident that the ascorbate in the slurries was protected against

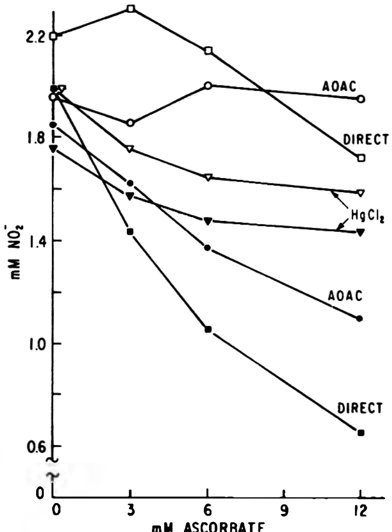

Figure 4. Mean values (n = 6) illustrating 3-factor interaction of preparation method, colorimetric reagent, and ascorbate. □, Direct reading; ○, AOAC; △, $HgCl_2$. Solid symbols, SAN; open symbols, SAA.

destruction during the heating period of the AOAC procedure. Meat contains residual reductants produced during glycolysis, which apparently protect the ascorbate against oxidation at higher temperatures.

The presence of residual ascorbate in the samples leads to an artifact in the measurement of nitrite. In Figure 4, the mean value curve for the AOAC/SAA combination shows no apparent loss in nitrite with increasing ascorbate. The experimental data in Table 2 also show several examples of this same lack of dependence, e.g., the AOAC/SAA values for the cooked, no-salt samples. This phenomenon, a result of the combined effects of ascorbate on nitrite loss and enhancement/decrease of pigment formation, explains an apparent disagreement in the literature. Sen and McPherson (14) reported a 1.5% loss of nitrite with 2 μg/mL ascorbate, while Adriaanse and Robbers (1) reported a 49% loss at the same ascorbate concentration. This difference in percent loss may be explained by the use of the sulfanilic acid/*N*-(1-naphthyl)-ethylenediamine combination by Sen and McPherson and the use of sulfanilamide and 1,7-Cleve's acid (8-amino-2-naphthalenesulfonic acid) by Adriaanse and Robbers.

Conclusions

The results of this study show that the amount of nitrite measured in a given sample is a function of its prior treatment, that is, whether heat-processed or not, its composition, the way it is prepared for analysis, and the reagent used for nitrite measurement, all of which factors interact. Under these conditions it is difficult to establish a true or correct value for the amount of nitrite in the sample, unless some criteria are available to show whether a specific interference or interaction has been eliminated. We have established such a criterion in the differential effect of residual ascorbate on the production of pigment from the 2 reagent combinations, SAA/NED and SAN/1-NA. Application of this criterion to our data indicates that the use of mercuric chloride is superior to the AOAC procedure for removing ascorbate from meat systems, but we do not specifically recommend the use of $HgCl_2$ as there may be other procedures as effective and more environmentally acceptable.

A second criterion is that the amount of pigment produced by a given set of colorimetric reagents should be the same in both standards and substrates. This can be achieved either through appropriate sample preparation to remove interfering compounds or by selection of appropriate reagents. The second alternative is superior to the first because it requires less handling of the sample. By this reasoning, SAN is preferred to SAA because the amount of pigment produced from the former does not depend on the salt concentration. Most official methods use SAN (4, 7, 8), but SAA has been used in recent studies (3, 6).

We recommend the use of the 2 reagent combinations, sulfanilic acid/*N*-(1-naphthyl)-ethylenediamine and sulfanilamide/1-naphthylamine, as a quick method for detecting residual ascorbate while determining nitrite. We also recommend the use of sulfanilamide as a Griess colorimetric reagent because pigment production from it is insensitive to chloride concentrations.

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INDUSTRIAL CHEMICALS

Liquid and Gas Chromatographic Analysis of Diethyl Phthalate in Water and Sediment

WILLIAM R. PAYNE, JR, and JACQUELYN E. BENNER
Environmental Protection Agency, Environmental Research Laboratory, Athens, GA 30613

Diethyl phthalate was determined in water and sediment by high performance liquid chromatography (HPLC) and in water by gas-liquid chromatography with electron capture detection (GLC-ECD). Water samples were extracted with hexane, using a high-speed homogenizer-ultrasonic apparatus and a test tube mixer. Sediments were Soxhlet-extracted using acetonitrile. For HPLC, diethyl phthalate was determined in normal phase mode using a Zorbax-CN column, a 2% isopropanol-hexane solvent system, and a UV variable wavelength detector. For GLC-ECD, a 3% SE-30 Gas-Chrom Q column with a ^{63}Ni electron capture detection system was used. Recoveries from fortified samples ranged from 93.9 to 98.0% for water at 0.01-0.50 ppm, and from 90.0 to 93.6% for sediment at 0.2-2.0 ppm.

The EXposure Analysis Modeling System (EXAMS), a computerized system for analyzing the fate of toxic substances in aquatic environments, is currently under development by the Environmental Protection Agency (L. Burns and M. Cline, EPA, Athens, GA). Tests to compare predictions from EXAMS with experimental data from controlled laboratory ecosystems required an analytical method for diethyl phthalate (DEP) in water and sediment. The research dictated that the method be applicable to large numbers of samples with emphasis on reducing the time between sampling and extraction procedures.

DEP is widely used as an insect repellent, for plasticizing cellulose esters, in propellant formulations, and in the manufacture of fragrances. The production of phthalate esters in the United States has increased at a steady rate from 300 million pounds in 1960 to over 1 billion pounds in 1975. The annual production is expected to increase to approximately 10 billion pounds by the turn of the century (1).

Peakall (2) reviewed the occurrence, biological activity, toxicology, and accumulation factors for phthalates in the environment. The review pointed out that data are lacking to adequately assess the environmental risk and to predict the point of occurrence of maximum residue levels.

Several authors have addressed the analytical procedures and problems in the analysis of phthalates. Fishbein and Albron (3) reviewed the analytical methods, biological properties, stability, and episodes of phthalate occurrence in the enviornment. Giam et al. (4-6) and Ishida et al. (7) considered the problem of background contamination from phthalates present in commercial laboratory reagents, glassware, and equipment.

The use of high performance liquid chromatography (HPLC) was proposed by Mori (8) for the determination of phthalates in river water and by Persiani and Cukor (9) for the determination of phthalates in industrial effluents and biological samples.

Corcoran (10), Thomas (11), and Norwitz and Apatoff (12) proposed gas chromatographic (GLC) methods using the electron capture detector (ECD) for phthalates. The detector, which responds to many classes of compounds with an affinity for electrons, usually exhibits high background because of interference from coextractives in environmental samples with inadequate or no cleanup. Time-consuming cleanup and isolation techniques cannot be omitted in many samples, especially sediments, if the electron capture detector is to be used for determination. The problem of background interferences can be lessened by the use of HPLC. Less sample preparation and cleanup is required by taking advantage of the wide selection of column types, mobile phases, and detectors available, such as the variable wavelength UV detector. For example, the molar absorption coefficient at 224 nm, the maximum absorption wavelength for DEP, is approximately 10 times that of the standard fixed wavelength, 254 nm, thus providing some degree of selectivity for this detector.

METHOD

Apparatus and Reagents

(**a**) *High performance liquid chromatograph.*— Tracor Model 900-78S chromatograph with a

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970A variable wavelength UV detector and Valco UCLI closed loop injection system. Column was Zorbax-CN (E. I. du Pont de Nemours & Co., Inc., Wilmington, DE), 25 cm × 4.6 mm id, with Whatman Co:Pell PAC guard column and mobile phase of 2% isopropanol-hexane at 1.0 mL/min and 350–400 psi.

(b) *Gas-liquid chromatograph.*—Tracor Model 222Q chromatograph with ^{63}Ni electron capture detector operated in the DC mode. Column was borosilicate glass, 8 ft × 1/4 in. od cut for off-column injection and packed with 3% SE-30 on 80–100 mesh Gas-Chrom Q. Operating conditions: nitrogen flow rate, column 50 mL/min, purge 30 mL/min; temperatures (°C)—column, 200; detector, 275; injection port, 240; transfer line, 275; Westronics MT-22 recorder 30 in./h.

(c) *Homogenizer.*—Brinkmann Polytron PT-10-35 with PT-20ST probe generator.

(d) *Test tube mixer.*—Vortex Genie K-550-G.

(e) *Soxhlet apparatus.*—Pyrex 3840.

(f) *Solvents.*—Acetonitrile, hexane, isooctane, isopropanol—distilled in glass, all solvents HPLC grade (Burdick & Jackson Laboratories, Inc., Muskegon, MI).

(g) *Diethyl phthalate.*—(Aldrich Chemical Co., Milwaukee, WI). Weigh 50 mg and place into 100 mL volumetric ₮ flask and dilute to volume with final extracting solvent. Stock standards are stable if stoppered tightly, sealed, and protected from light under refrigeration.

HPLC Determination

(a) *Water.*—Transfer 300.0 mL sample to 500 mL reagent bottle that contains 100 mL hexane. Extract samples, using Polytron homogenizer 2 min at 25 000 rpm. Transport bottles to laboratory for following additional extractions: Transfer contents of bottles to 1 L separatory funnels with hexane, allow phase separation, and transfer water layer to second 1 L separatory funnel. Collect hexane from first extraction in 400 mL beaker. Extract water in 1 L separatory funnel two more times, using homogenizer and 100 mL and 50 mL portions of hexane. Add these extracts to first extract in 400 mL beaker and concentrate combined hexane extracts on water bath to ca 5–8 mL. Transfer quantitatively with hexane to graduated (calibrated) 15 mL conical test tubes and seal with ₮ stoppers.

(b) *Sediment.*—Clean glass wool and filter papers (Whatman No. 42, 15 cm) by placing them in large Soxhlets and extracting 4 h with hexane. Transfer glass wool and filter papers to beakers and place on steam bath under hood to evaporate hexane. Spread filter papers on acetone-rinsed aluminum foil and dry 1 h in 120°C oven. Place in desiccator, cool 5 min, remove one at a time, number, and record tare weight. Place Büchner filtering funnel (127 mm od) on side-arm filtering flask with filter-vac disc. Prewet filter paper and shape into Büchner funnel, leaving small rim of filter paper turned up around sides of funnel. Seat paper with low vacuum, add wet sediment, and rinse with ca 10 mL distilled water to form sediment pad. Release vacuum, remove pad and filter, and gently fold in fluted form. Place sediment and filter into Soxhlet extractor containing small plug of glass wool at the bottom and add small plug of glass wool to top of sample. Extract 4 h with acetonitrile. Siphon over any acetonitrile remaining in upper Soxhlet after extraction and rinse and siphon several times, collecting siphonings in beaker. Remove top glass wool plug, place pad in small beaker, and dry overnight in 120°C oven. Remove from oven to desiccator, cool for 30 min, weigh pad, and subtract tare of filter paper to obtain total net dry weight.

Quantitatively transfer acetonitrile extract to beaker containing previous rinses and concentrate to ca 100 mL. Transfer quantitatively to 2 L separatory funnel containing a 10× dilution of distilled water. Extract 3 times with 100, 100, and 50 mL portions of hexane, collecting all extracts in 400 mL beaker. Concentrate combined extracts on water bath to 5–8 mL, transfer quantitatively to 15 mL graduated (calibrated) conical test tube, and seal with ₮ stopper. Record volume in graduated tubes for water and sediment extracts. Mix thoroughly and, using 50 μL syringe, inject into 20 μL HPLC sample loop. DEP concentration is determined by peak height comparison of sample and DEP reference standard.

GLC-ECD Determination for Water

Transfer 2.0 mL sample to 15 mL ₮ conical test tube and add 1.0 mL isooctane. Experimental data on 6 sample:solvent ratios show that sample size may be varied with good toxicant recovery if the sample:solvent ratio is 2:1. Extract by mixing stoppered sample tubes 2 min on vortex mixer at 1000–1200 rpm. Let solvent-water layer separate and inject 3–8 μL of top isooctane layer into gas chromatograph. DEP concentration is determined by comparing peak heights of sample with DEP standard.

Results and Discussion

Fortified ecosystem water samples were chromatographed on a Zorbax-ODS methanol-

Table 1. Recoveries of DEP from fortified samples of water and sediment

Substrate	Amt added, ppm	No. of analyses	Recovery, %
Water (300 mL)	0.01	7	94.6 ± 1.6
HPLC	0.02	7	93.9 ± 3.8
	0.05	6	94.8 ± 2.1
	0.10	14	96.3 ± 2.7
Sediment (20.0 g)	0.2	4	93.6 ± 2.8
HPLC	0.4	4	90.0 ± 4.3
	0.8	4	90.6 ± 2.8
	2.0	12	92.5 ± 1.8
Water (2.0 mL)	0.02	6	94.9 ± 2.1
GLC-ECD	0.05	6	94.6 ± 2.3
	0.10	11	98.0 ± 2.3
	0.50	6	96.4 ± 2.2

water reverse phase HPLC system to explore the feasibility of direct sample injection. The lower limit of detection for this system was in the range of 1.50–0.50 ppm, which was higher than the anticipated concentration of DEP in the model ecosystem.

To concentrate the toxicant for HPLC, several solvents were tested for liquid-liquid extraction. Analytical results showed that a DEP-solvent mixture could not be evaporated completely to dryness and redissolved in methanol for the reverse phase system without subsequent loss of toxicant. Solutions of DEP in methanol, hexane, and methylene chloride evaporated on a water or dry bath at 80°C to 0.5–1.0 mL, transferred to conical test tubes, and evaporated to dryness with a stream of dry air, showed losses ranging from 10 to 25%. Hexane, which was selected as the extracting solvent, was concentrated to 5–8 mL and transferred to a 15 mL graduated ፣ centrifuge tube for HPLC determination.

Several HPLC systems compatible with hexane were investigated and a normal phase Zorbax-CN column, using isopropanol hexane mobile phase, was selected. The detection limit for HPLC using this system was 20 ng DEP at 25% full scale deflection (FSD). The recovery from water samples fortified with DEP-distilled water standards is shown in Table 1. Figure 1 demonstrates the response of DEP at 224 nm compared with that at 254 nm, and chromatograms of DEP in ecosystem samples are shown in Figure 2.

HPLC was the method of choice because it provided a technique for DEP in water and sediment with little or no cleanup. But for water analysis, a minimum sample volume of 300 mL was required to achieve method sensitivity. The GC-ECD method was used as a confirming technique for DEP in water, and this simple, rapid method would be applicable to situations where sample volume is limited. No major difficulty was experienced in the evaluation of the resulting chromatograms (Figure 3). However,

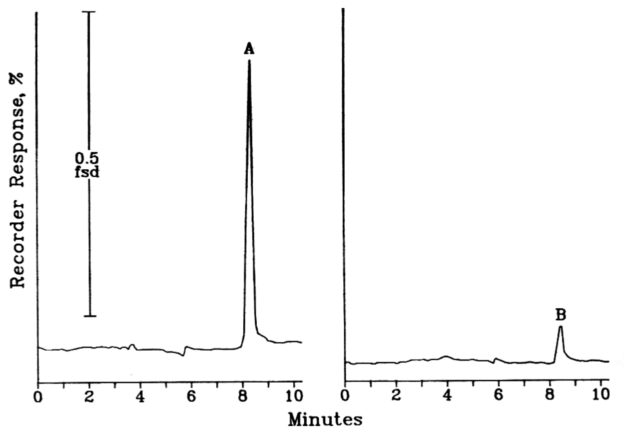

Figure 1. Liquid chromatograms of DEP response at 224 nm (A) and 254 nm (B).

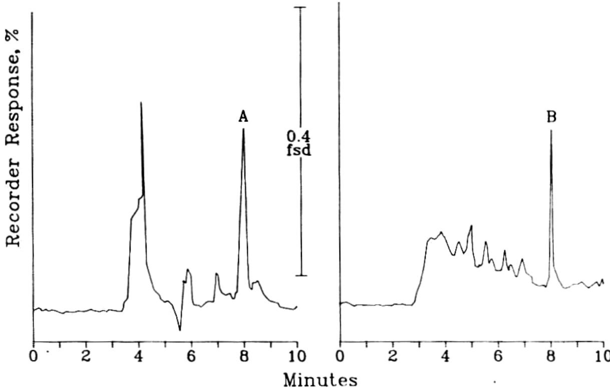

Figure 2. Liquid chromatograms of DEP in ecosystem water at 0.05 ppm (A) and sediment at 2.0 ppm (B).

it is probable that some environmental water samples would require cleanup using techniques such as the deactivated (3% water) Florisil procedure described by Giam et al. (4, 6). The detection limit for the ECD was 0.5 ng DEP at 20% FSD and recovery from fortified small volume water samples is shown in Table 1. Attempts to analyze the sediment extracts by ECD after acetonitrile–hexane partitioning were not successful, and the main disadvantage of this method is that many environmental samples (e.g., large water volumes, sediments) must undergo rigorous cleanup before ECD determination. The continuous injection of samples with inadequate cleanup can contaminate the GLC column and the EC detector, causing tailing, baseline noise, drift, and loss of sensitivity. However, the potential application of this method to water samples from small model ecosystems as a screening and/or confirming technique for DEP should not be overlooked.

To minimize the problem of interferences from other phthalates such as diethyl hexyl phthalate (DEHP), a common laboratory contaminant, glassware, filters, and reagents were cleaned and extracted with high purity solvents and/or heat-treated (6). As a result, procedural blanks did not show any significant background which interfered with DEP. However, the ability of this HPLC system to resolve DEP and DEHP is illustrated in Figure 4.

Distilled water standards of DEP should be kept under refrigeration when not in use. DEP-water standard held at ambient temperature

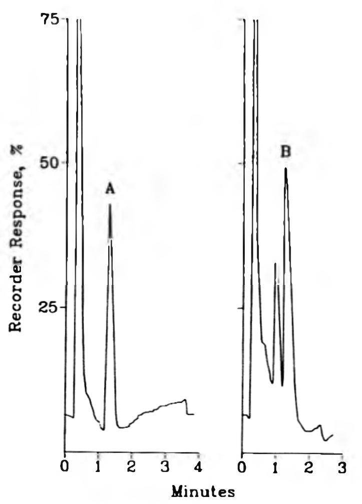

Figure 3. Gas chromatograms of 2.0 ng DEP standard (A) and DEP in ecosystem water at 100 ppb (B).

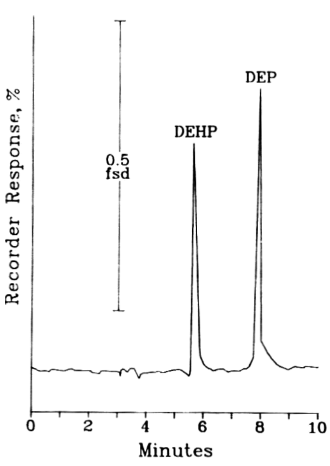

Figure 4. Liquid chromatograms of standard mixture containing DEHP (100 ng) and DEP (100 ng) in hexane at 224 nm.

under laboratory fluorescent lighting decreased in concentration by 20% in 4 days and 70% in 12 days. No DEP could be detected after 14 days. DEP loss in distilled water in the absence of light was somewhat slower. Water standards of DEP held under refrigeration showed no loss after 60 days.

The water samples for HPLC determinations were extracted on-site immediately after sampling, and the GLC water samples were refrigerated and extracted within 1-2 h after sampling. There were no significant differences in the final results. However, the need to minimize the lag time from sampling to extraction was demonstrated when a water sample from the ecosystem was analyzed, held at ambient temperature, and re-analyzed in 24 and 48 h. These re-analyses revealed DEP losses of 64 and 89%, respectively, from the initial concentration. When it is not possible to extract the samples promptly, the need for immediate refrigeration cannot be overemphasized. Selected stored samples should be reanalyzed at intervals and fortified control samples should be held under the same conditions to determine the effects of cold storage.

The acetonitrile-hexane partitioning procedure provided adequate cleanup for HPLC determination in all sediment samples analyzed. The recovery from sediment samples fortified with DEP-distilled water standards is shown in Table 1. If time is not a factor, additional cleanup is recommended in order to prolong the life of the HPLC column, and detector cell windows.

The use of the Polytron ultrasonic apparatus for the extraction of DEP from sediments was also investigated and the difference in recoveries between the Polytron and Soxhlet techniques was small. The ultrasonic procedure is much faster than the Soxhlet but the stainless steel generator probe wears rapidly because of the abrasive action of the sediment. Johnsen and Starr using this apparatus for soil extractions reported that replacement was necessary after 150-200 extractions (13). The current replacement cost is $600. The Soxhlet technique, although lengthy, requires minimum technician attendance after the initial set up until the extraction period is completed.

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EXTRANEOUS MATERIALS IN FOODS AND DRUGS

Determination of Internal Insect Infestation of Wheat: Collaborative Study

RICHARD L. TRAUBA
Food and Drug Administration, 240 Hennepin Ave, Minneapolis, MN 55401

Collaborators: L. Glaze; E. D. McGary; W. D. Palmer; J. T. Quaife; J. Strozier; A. Whiteman

An improved method has been developed for determining internal insect infestation of wheat kernels. The method involves acid hydrolysis of cracked wheat, wet sieving to remove the acid, transferring to a 2 L Wildman trap flask, deaeration by boiling, and treatment with Tween 80-Na_4EDTA. Insects are extracted with light mineral oil. Reports from 6 collaborators showed that recovery was 95.92% for adult insect heads and 97.22% for larvae by the proposed method as compared with 87.05% and 6.12%, respectively, by the official method. The method has been adopted official first action.

The present official AOAC method for internal insect infestation in grain, **44.037** (13th Ed.), produces an excessive amount of plant debris which floats and is trapped off with the heptane layer, resulting in the use of 5–10 filter papers per 100 g sample.

In the proposed method, a 50 g sample is used. The sample is hydrolyzed with HCl to break down the starch. The hydrolyzed sample is placed on a sieve and washed with water to remove the acid to prevent any chemical reaction between HCl and Na_4EDTA. The material is then transferred to a 2 L trap flask with 40% isopropanol, deaerated, and treated with Tween 80–Na_4EDTA solution to prevent the plant material from floating with the oil. Mineral oil is added and the contents are mixed. The trapped-off oil layer contains very little plant debris, resulting in 1 or 2 clean filter papers.

Method **44.037** specifies grinding the sample in a cutting-type mill set at 0.061 in. In the 12th edition of *Official Methods of Analysis*, a Labconco mill or equivalent was suggested but this mill is no longer manufactured. We found that an electric coffee grinder (Norelco "dial-a-bean" Model HB5115, North American Philips Corp., Consumer Products Div., PO Box 5845, New York, NY 10017), when set at its coarsest grinding position, gave cracked wheat equivalent in particle size to that obtained from the Labconco mill set at 0.061 in.

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This report of the Associate Referee was presented at the 95th Annual Meeting of the AOAC, Oct. 19–22, 1981, at Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and Committee F and adopted by the Association. See *J. Assoc. Off. Anal. Chem.* **65**, March issue (1982) for detailed reports.

Collaborative Study

Six collaborators examined six 100 g spiked samples of previously cracked wheat by the official method and the same number of spiked 50 g samples by the proposed method. The spikes in all samples consisted of the adult heads of 10 *Tribolium* sp., 10 *Sitophilus oryzae*, 10 *Rhyzopertha dominica*, and 5 whole *Sitophilus oryzae* larvae.

Wheat—Internal Insect Infestation
Official First Action

Reagent

Tween 80-Na_4EDTA [(ethylenediamine)-tetraacetic acid tetrasodium salt] premix soln.—Measure 420 mL 40% isopropanol in 500 mL graduate. Add 80 mL Tween 80 (polysorbate 80) to 100 mL g-s graduate. Invert 100 mL graduate over 2 L glass beaker and drain briefly. Rinse 100 mL graduate with several portions of the 420 mL 40% isopropanol, pouring each rinse into beaker. Add rest of 40% isopropanol to beaker, add mag. stirring bar, and start mag. stirrer. Add 10 g Na_4EDTA to beaker while stirring rapidly. Add 500 mL 40% isopropanol and stir until uniform. Mixed reagent stored in g-s flask is stable 1 week.

Preparation of Sample

Mix grain by passing 6 times through Jones sampler, recombining sepns before each pass. Sep. slightly >50 g and weigh 50 g. Transfer weighed sample, small amt at a time, to 5 or 8 in. No. 12 sieve, and with stiff bristle brush, work insects thru sieve as completely as possible.

Grind screened sample in cutting-type mill set

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Table 1. Collaborative results for recovery of adult insect heads (30 added) from wheat kernels by official and proposed methods

	Official						Proposed					
Coll.	A	B	C	D	E	F	A	B	C	D	E	F
1	26	27	27	28	27	26	30	24	30	30	30	30
2	28	29	27	27	29	29	29	24	27	30	30	25
3	23	25	22	20	24	22	30	30	28	30	30	30
4	27	30	29	29	29	29	29	30	29	30	30	29
5	23	24	27	26	27	27	28	29	20	30	26	22
6	22	26	25	26	25	23	30	29	29	30	29	30
Av., %			87.05						95.92			
SD												
Repeatability			1.374						1.987			
Reproducibility			2.634						2.071			
CV, %												
Repeatability			5.26						6.87			
Reproducibility			10.09						7.20			

at 0.061 in. (An electric coffee grinder, Norelco "dial-a-bean" Model HB5115, North American Philips Corp., or equiv., using coarsest grinding position, can be used.) Dry damp or tempered grain in forced-draft oven 1 h at 70–80° or 2 h in oven without draft.

Isolation

Transfer cracked grain, including any residue in mill, to 2 L glass beaker contg mag. stirring bar, **44.002(p),** and mixt. of 600 mL H_2O + 50 mL HCl. Stir *gently* while boiling 15 min on hot plate.

Transfer sample to No. 100 sieve, **44.002(u),** with gentle stream of hot tap H_2O. Wash material on sieve with *very gentle* stream of hot (55–70°) tap H_2O until washings show no acidity when tested with blue litmus paper.

Add mag. stirring bar, **44.002(p),** to 2 L trap flask, **44.002(i)**(3). Place wide-stem funnel in flask opening and quant. transfer residue on sieve to flask with 40% isopropanol. Add 40% isopropanol to total vol. of 800 mL.

Clamp stirring rod so stopper or wafer is above liq. in flask. (Trap flask may stand overnight at this point.) Stir *gently* while boiling 7 min ± 10 s on mag. stirring hot plate. Remove flask from hot plate and wash down sides with min. of 40% isopropanol and immediately add 100 mL Tween 80–Na_4EDTA soln slowly down rod. Hand-stir *gently* 1 min and let stand 3 min.

Add 50 mL mineral oil, **44.003(y),** down stirring rod. Stir mag., **44.004(b),** 5 min on cool mag. stirrer, and let stand 3 min.

Fill flask with 40% isopropanol, added slowly down stirring rod to avoid mixing or agitation of flask contents, and let stand 20 min *undisturbed.* Trap off, rinsing neck of flask with 40% isopropanol, and add rinse to trappings in beaker.

Add 35 mL mineral oil to flask and hand-stir 1 min. Clamp stirring rod so stopper or wafer is at midpoint of flask. Let stand 5 min, spin stirring rod to free settlings from stopper or wafer, and adjust oil level with 40% isopropanol to ca 1 cm above fully raised stopper. Let stand *undisturbed* 15 min. Trap off, and combine trappings in beaker. Rinse neck of flask well with isopropanol, adding rinsings to beaker. Transfer trappings to ruled filter paper, rinsing beaker well with isopropanol. Examine papers at 15X, counting only whole insects, insect heads, cast skins, and head capsules.

Results and Discussion

Tables 1 and 2 show the recoveries of the spiked elements. Average recoveries of adult insect heads by the proposed and the official methods were 95.92 and 87.05%, respectively, for adult insect heads and 97.22 and 6.12% for larvae. The 2 methods give comparable average recoveries and precision for adult insect heads, but the proposed method gives better results for the recovery of larvae.

The number of needed extraction papers averaged 6.2/sample for the official method, and 1.5/sample by the proposed method. The average time required for analysis was 218 min/sample by the official method and 162 min/sample by the proposed method.

All collaborators preferred the proposed method to the official method, and reported that the extraction papers produced were cleaner and easier to read.

Table 2. Collaborative results for recovery of larvae (5 added) from wheat kernels by official and proposed methods

	Official						Proposed					
Coll.	A	B	C	D	E	F	A	B	C	D	E	F
1	0	0	0	0	0	0	5	5	5	5	5	5
2	1	0	1	2	1	1	5	5	4	5	5	5
3	0	1	0	1	0	0	5	4	5	5	5	5
4	0	1	1	0	0	0	5	5	5	5	5	5
5	0	0	0	0	1	0	4	5	5	5	5	5
6	0	0	0	0	0	1	5	4	4	5	5	5
Av., %			6.12						97.22			
SD												
Repeatability			0.428						0.358			
Reproducibility			0.539						0.358			
CV, %												
Repeatability			139.87						7.37			
Reproducibility			176.14						7.37			

Recommendations

The proposed method for the determination of internal insect infestation of wheat kernels is recommended for adoption as official first action. The proposed method produces high recoveries for both adult and larval whole or equivalent insects, and clean extraction papers. No toxic solvents, which need special precautions, are used.

Acknowledgments

The author expresses his appreciation to the following collaborators, all of the Food and Drug Administration, for their work and comments: Larry Glaze, Washington, DC; Eddie D. McGary, Kansas City, MO; Wayne D. Palmer, Minneapolis, MN; John T. Quaife, Minneapolis, MN; Jennifer Strozier, Atlanta, GA; Al Whiteman, Chicago, IL; and to Foster D. McClure, FDA, Washington, DC, who did the statistical study.

FLAVORS AND NONALCOHOLIC BEVERAGES

Ion Pairing High Pressure Liquid Chromatographic Determination of Amaranth in Licorice Products

WILLIAM J. HURST, JAMES M. MCKIM, and ROBERT A. MARTIN, JR
Hershey Foods Technical Center, 1025 Reese Ave, Hershey, PA 17033

A high pressure liquid chromatographic method is described for the determination of amaranth (FD&C Red No. 2; Red No. 2) in licorice products. The Red No. 2 is extracted with a basic buffer solution, cleaned up on a Sep-Pak column, chromatographed on a reverse phase column in the ion pairing mode, and detected at 254 nm. The procedure is time-conservative with accurate and precise results. Recovery data ranged from 93 to 104%, and coefficients of variation were less than 4% for standards and samples.

Amaranth was one of 7 dyes permitted for use in foods by the Food and Drug Act of 1906. Amaranth (FD&C Red No. 2; Red No. 2) was used for many years, but in the early 1970s several studies were conducted which resulted in the delisting of Red No. 2 in the United States by 1976 and then termination of listing by 1980 (1). This color, although delisted in the United States, is allowable in Canada, Sweden, West Germany, Japan, and 9 countries of the European Economic Community (2).

Amaranth

FD&C dyes are water-soluble compounds consisting of 4 classes (3): azo dyes, triphenylmethane dyes, fluorescein types, and sulfonated indigo. FD&C Red No. 2 is an azo dye. Compounds in this class have good water solubility due to the sulfonic acid groups, which also reduce oil solubility.

FD&C dyes in food have traditionally been determined by extraction of the dyes followed by column chromatography and spectrophotometric determination (4). Other techniques such as thin layer chromatography and differential pulse polarography have either supplemented or complemented the column chromatographic method (5–8). High pressure liquid chromatography (HPLC) has been used to separate dyes for determining uncombined intermediates (4) and has recently been used to analyze dyes in many matrixes (9–14).

In this method, the determination of FD&C Red No. 2 in a licorice matrix is outlined.

Experimental

HPLC Apparatus

The HPLC system used in this study included the M6000A solvent delivery system (Waters Associates) and a Model 440 absorbance detector at 254 nm (Waters Associates). The HPLC column was μBondapak C_{18}, 4.0 × 30 cm (Waters Associates), and the HPLC injection valve was a Model 7120 HPLC injector (Rheodyne). Data were quantitated using a digital integrator, ITG-4A (Shimadzu Scientific).

Reagents

The 5 μg/mL Red No. 2 standard was diluted to final concentration with LC grade water. The HPLC mobile phase was a 1 + 6 mixture of isopropanol (Baker HPLC grade) and 0.005M tetrabutylammonium hydrogen sulfate (Sigma Chemical Co.), pH = 7. Other reagents used were boric acid buffer (0.5M), 1% α-amylase, 10% NaOH, 1N HCl, and Sep-Pak elution solvent water–methanol–NH_4OH (50 + 50 + 7).

Sample Preparation

All samples were ground or grated to a uniform size in a Mouli grater.

Procedure

Five g sample was weighed into a 50 mL Sorvall blender cup with 30 mL boiling LC water. This was blended 3 min at high speed, using the Sorvall blender. The suspension was quantitatively transferred to a 250 mL beaker, 1 mL of 1% α-amylase was added, and the resulting mixture was incubated 0.5 h at 37°C. The mixture was cooled to room temperature, 10 mL boric acid buffer was added, and pH of solution was adjusted to 10 ± 0.1 with 10% NaOH. The solution was quantitatively transferred to a 100 mL volumetric flask and diluted to volume with water.

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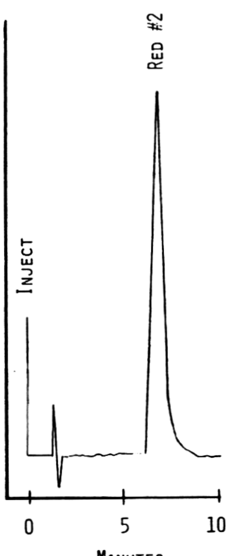

Figure 1. Chromatogram of Red No. 2 standard.

This solution was centrifuged and 5 mL supernate was withdrawn and placed in a beaker with 500 μL concentrated HCl.

C_{18} Sep-Pak cartridge was prewetted with isopropanol followed by 10 mL 1N HCl. The acidified sample was transferred to the cartridge, which was washed with three 10 mL portions of 1N HCl. The Red No. 2 was eluted with 20 mL elution solvent, the pH was adjusted to 6–7 with CH_3CO_2H, and the extract was diluted to 50 mL with water.

HPLC Determination

Fifty μL extract was injected into the HPLC system and compared with 50 μL injections of standard. Chromatograms of standard and sample are presented in Figures 1 and 2.

Results

Duplicate recovery studies were done at 5 levels of addition with recoveries ranging from 93.5 to 104.0%. Data are summarized in Table 1.

Precision of standard and sample analyses was also studied. Five analyses of a 250 ng standard gave a coefficient of variation of 3.9%. Five analyses of a 300 ng sample gave a coefficient of variation of 3.87%.

The method exhibits good linearity from 125 ng to 1.125 μg injected.

The results indicate that the method exhibits good accuracy and precision over a 10-fold range. The method is time-conservative and straightforward. Furthermore, it uses the C_{18} Sep-Pak cartridge for rapid sample extract cleanup. The chromatography separates Red No. 2 from Red No. 40, Yellow No. 5, and Yellow No. 6 (11). This method allows the analyst a rapid means to identify and quantitate Red No. 2 in a wide variety of food products.

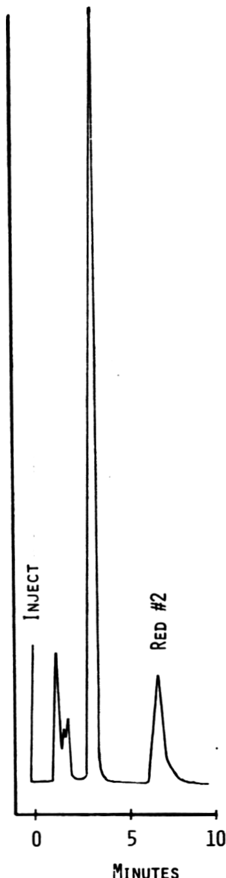

Figure 2. Chromatogram of licorice extract.

Acknowledgments

The authors thank members of the Analytical Research Group for continuing support, Y&S

Table 1. Recovery of amaranth from licorice by ion pairing high pressure liquid chromatography

Amount added, μg	Amount recovered, μg	Recovered, %
200	187.7	93.9
400	379.4	94.9
600	624.4	104.0
800	747.8	93.5
1000	952.4	95.4
Av.		96.3

Candies of Canada for licorice samples, and Hershey Foods Corp. for the opportunity to publish this research.

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DAIRY PRODUCTS

Estimation of Lactose Hydrolysis by Freezing Point Measurement in Milk and Whey Substrates Treated with Lactases from Various Microorganisms

SHIH-LING YEH CHEN, JOSEPH F. FRANK, and MORRISON LOEWENSTEIN
University of Georgia, Animal and Dairy Science Department, Athens, GA 30602

β-Galactosidase concentrates obtained from several microorganisms were used to hydrolyze skim milk, low fat (2%) milk, sweet whey, acid whey, acid whey permeate, and acid whey concentrate. Among acid substrates, the freezing point depression for each 1% lactose hydrolyzed was the greatest with the lactase from *Aspergillus niger* (0.0501°H); among neutral substrates, the depression was greater in sweet whey (0.0495°H) and lesser in low fat milk (0.0445°H). All data were statistically significant. The average freezing point depression for each 1% lactose hydrolyzed was 0.0468°H (range 0.0436-0.0501°H). Oligosaccharides formed in the lactose hydrolysis, inconsistent freezing point readings of the cryoscope at the low freezing points measured, and protease contamination in some lactases may affect the precision of freezing point determination. Hydration and volume of non-protein components in commercial enzymes, unstable color complex formed by lactose in methylamine solution, and difficulty in the use of methylamine solution might cause variations in determination of lactose by the analytical procedure. These factors can be eliminated or minimized. This method is the simplest and quickest estimation of lactose hydrolysis, and it offers great accuracy and consistency.

When lactose is hydrolyzed by β-galactosidase, it is split into galactose and glucose. The increase in the molarity of the soluble compounds lowers the freezing point of the solution. Lactose and chlorides contribute nearly 77% to the freezing point depression in cow's milk (1); therefore, depression in the freezing point caused by lactose splitting can be correlated to the degree of lactose hydrolysis.

Baer et al. (2) introduced the cryoscopic method to determine lactose hydrolysis in neutralized acid whey and lactose solution treated with β-galactosidase from *Saccharomyces lactis*; Zarb and Hourigan (3) determined lactose hydrolysis in reconstituted whey (0–5% lactose) and β-galactosidase from *Escherichia coli*.

Baer et al. (2) reported that there was a direct relationship between freezing point depression and lactose hydrolysis: For each 1% lactose hydrolyzed, the freezing point was lowered approximately 0.050°H in neutralized acid whey and lactose solution treated with β-galactosidase from *S. lactis*. This method may offer a simple, quick, and accurate estimation of lactose hydrolysis in dairy products and whey substrates. In this study, β-galactosidase concentrates obtained from different microorganisms were used to treat different substrates to determine if a linear relationship exists between freezing point depression and lactose hydrolysis, and if the freezing point is lowered approximately 0.050°H for each 1% lactose hydrolyzed in all those substrates treated with different enzymes.

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Experimental

Materials

(a) *Enzymes.*—β-Galactosidase concentrates provided by 5 different companies were used. The source and properties of these enzymes (4–7) are listed in Table 1.

(b) *Milk.*—Milk and skim milk (SM) were obtained from the University of Georgia creamery. Low fat milk (LFM) was prepared by mixing 1200 mL milk with 800 mL skim milk.

(c) *Whey.*—Acid whey (AW) from cottage cheese was obtained from the University of Georgia dairy manufacturing plant. Acid whey concentrate (AWC) and permeate (AWP) were prepared by processing acid whey of the same batch through the ultrafiltration system (Abcor 5-HFM-180-S-S-W tubular polysulfonate membrane system). Approximately 60 gal. whey was ultrafiltered to obtain 15 gal. whey concentrate. Sweet whey (SW) was prepared by rennet precipitation of whole milk; precipitated protein and entrapped solids were removed by decantation of the clear whey. Whey samples were stored at −20°C after preparation or collection.

Methods

Composition.—All substrates (AW, AWP, AWC, SW, SM, LFM) were analyzed for the following

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Table 1. Source and properties of β-galactosidase preparations tested

Company[a]	Source	Optimum pH	Optimum temp., °C	Activity[b]	
A	*Aspergillus oryzae*	4.5–5.0	55	14 000	FCC LU/g
B	*A. oryzae*	4.5–5.0	55–60	15 000	mU β-galactosidase/mg
C	*A. oryzae*	4.5–5.0	55	5 000	ONPG U/g
D	*A. niger*	4.5	58	6 667	FCC LU/g
E	*Kluyveromyces fragilis*	6.5–7.0	40	750	LAU/mL

[a] Companies producing β-galactosidase preparations listed in alphabetical order:
Enzyme Development Co., 2 Penn Plaza, Philadelphia, PA
Fermco Biochemics Inc., Elk Grove Village, IL
Gist-Brocades Fermentation Industries Inc., Des Plaines, IL
Miles Laboratories, Inc., Elkart, IN
Novo Laboratories Inc., Wilton, CT

[b] FCC LU means Food Chemical Codex Lactase Unit. One FCC LU is that activity which liberates 1 μmol *o*-nitrophenol from o-(*p*)-nitrophenol-D-galactopyranoside (ONPG) per minute at 37°C and pH 4.5.

One mU β-galactosidase corresponds to that enzyme quantity which liberates 1 μmol ONPG within 10 min at 30°C and pH 5.

LAU means lactase unit. One LAU is defined as the amount of enzyme that releases 1 μmol glucose per minute from 4.7 w/v lactose substrate under standard conditions (37°C, pH 6.5 milk buffer system).

One ONPG unit is defined as the activity to liberate 1 μmol *o*-nitrophenol per minute from 0.15% substrate solution of the ONPG at pH 6.5 and 30°C.

components: fat—Babcock test (8); total solids—AOAC **16.032** (9); ash—AOAC **16.035** (9); nitrogen—micro-Kjeldahl, AOAC **34.021** and **47.021** (9); titratable acidity—with 0.1N NaOH and phenolphthalein, expressed as % lactic acid (8); and pH—potentiometer with combination electrode (Orion Research Model 701/digital pH meter).

Lactose content in substrates was determined by the method of Nickerson et al. (10) in which zinc acetate phosphotungstic acid (ZAPT) reagent was used to precipitate protein and stop the enzyme reaction, and the red complex formed by lactose in hot alkaline methylamine solution was read in the spectrophotometer. Two modifications were made in the Nickerson et al. method. First, the dilution factor in sample preparation parts b and c was changed from 50 to 5, 10, or 25 for samples hydrolyzed over 20%, so that absorbance readings would fall in the range of 0.1 to 0.5 which is the most linear and sensitive range in the spectrophotometer. The selection of the dilution factor was based on the estimation of the degree of hydrolysis by freezing point depression measurement (Table 2). Second, sufficient amount (not necessarily 20 mL) of glacial acetic acid was used to provide a pH value of 4.6 in protein precipitation. The glacial acetic acid in ZAPT reagent was used to bring the pH to 4.6; therefore, the amount of glacial acetic acid needed should depend on the original pH of the substrates rather than on adding the same volume for all. A Coleman 111 Perkin-Elmer UV-VIS spectrophotometer was used to read absorbance at 540 nm. Since protein hydration and the volume of protein and fat influence the concentration of lactose in the filtrate, Grimbleby's formula (11) was used to correct the absorbance reading in the spectrophotometer:

Corrected reading = observed reading $\times$ [100 $- (P \times 0.84 + F \times 1.07)$]/100 where P and F are the percentages of protein and fat, respectively, in the tested samples.

Lactose hydrolysis.—Table 3 shows the 5 en-

Table 2. Modification of Nickerson et al. (10) method for lactose determination

		Modification on prepn of sample, mL					
		Part b			Part c		
FPD,[a] °H	Estimated hydrolysis, %	Filtrate	1N NaOH	H_2O	Filtrate	H_2O	Total diln factor
>0.190	90	2.5	2.5	5.0	5.0	0	5
0.160–0.190	70–90	1.5	1.5	9.0	5.0	0	10
0.050–0.160	20–70	0.5	0.5	9.0	5.0	0	25
<0.050[b]	20	0.5	0.5	9.0	5.0	5.0	50

[a] FPD means freezing point depression measured with cryoscope.
[b] No modification was made for samples hydrolyzed less than 20% (estimated by FPD < 0.050°H) and blanks.

Table 3. Enzyme, substrate, enzyme dosage, and conditions in lactose hydrolysis procedure

Enzyme	Substrate	pH of respective substrates			Temp., °C	Enzyme dosages of each substrate[a]		
A	AW, AWC, AWP	4.65	4.69	4.65	50	0.25	0.12	0.06
B	AW, AWC, AWP	4.65	4.69	4.65	50	0.08	0.04	0.02
C	AW, AWC, AWP	4.65	4.69	4.65	50	0.20	0.10	0.05
D	AW, AWC, AWP	4.65	4.69	4.65	50	0.70	0.40	0.10
E	SW, SM, LFM	6.50	6.50	6.50	40	1.0	0.50	0.20

[a] E was the only enzyme in liquid form; the unit of dosage is mL/200 mL substrate for E and g/200 mL substrate for others.

zymes, 6 substrates, and 3 enzyme dosages used in this research. The enzymes are listed according to manufacturer. Three enzyme dosages were used to treat each substrate to obtain a wide range of lactose hydrolysis percentage within 2 h. This permitted build-up of the regression line of freezing point depression (FPD) against degree of hydrolysis.

Freezing point determination.—Freezing points were determined with a Fiske MS™ cryoscope calibrated with the following aqueous NaCl standards from Fiske Associates: −0.422°H (−0.408°C), −0.540°H (−0.522°C), −0.621°H (−0.600°C), and distilled water (0.000°C). Cryoscope readout was given in millidegrees Horvet (°H).

Because of the inconsistency of the cryoscope readings at the low temperatures involved in these studies, the freezing point of the sample was read 3 times successively and only the third reading was used, to improve accuracy.

Results and Discussion

Composition of Milk and Whey Substrates

The composition, pH, and titratable acidity (TA) of milk and whey substrates are listed in Table 4.

Protein Content of Enzyme Concentrates

Protein content in the commercial enzyme preparations was as follows: A, B, C, D, E = 44.22, 26.10, 23.77, 13.04, 1.04% (% protein = %N × 6.25). The components other than protein in these concentrates could be materials which cause hydration and which volume may also affect the lactose concentration in the filtrate. These components were not taken into consideration in Grimbleby's formula (11).

The enzyme protein content in 200 mL substrate as well as that of the substrate itself were added as total protein (*P*), and all absorbance readings in lactose determination were corrected by Grimbleby's formula in the calculation of the correction factor in this study.

Effect of Enzyme Addition on Freezing Point

Freezing point depression resulting from addition of lactase concentrates was measured by subtracting freezing point of deionized water from that of the same solvent with enzyme solution added at each of 3 concentrations. Table 5 shows freezing point depression caused by the enzyme source.

Lactose Standard Curves

Methylamine hydrochloride absorbs moisture easily (12). In this study, it was dried 1 h at 100°C, cooled 10–20 min in a desiccator, and then weighed to make a 5% solution. In solution, methylamine exists as an ion ($CH_3NH_3^+$), but it might change to free molecular methylamine (CH_3NH_2) in the equilibrium system. Molecular

Table 4. Composition,[a] pH, and titratable acidity (TA) of milk and whey substrates

Substrate	Total solid, %	Protein,[b] %	Fat, %	Ash, %	Lactose, %	TA[c]	pH
AW	6.62	1.01	0.05	0.78	5.03	0.61	0.65
AWC	8.36	2.47	0.21	0.82	5.03	0.69	4.69
AWP	5.77	ND[d]	ND	0.78	4.82	0.58	4.65
SW	6.50	0.99	0.22	0.48	4.78	0.09	6.50
SM	9.84	3.59	0.01	0.78	4.99	0.19	6.50
LFM	11.76	3.59	2.15	0.76	5.28	0.18	6.50

[a] Means of duplicate or more determinations; numbers have been rounded.
[b] % Nitrogen × 6.38 = % protein.
[c] Expressed as % lactic acid.
[d] Not detectable at the sampling level of 0.5 g.

Table 5. Freezing point depressed (°H) by enzyme addition

Enzyme	1st dosage	2nd dosage	3rd dosage[a]
A	−0.013	−0.005	−0.002
B	−0.005	−0.003	−0.002
C	−0.015	−0.008	−0.004
D	−0.020	−0.013	−0.004
E	−0.089	−0.045	−0.018

[a] 1st dosage means the largest dosage used; and 3rd, the least.

methylamine is a gas. Although K_b (basicity constant) of $CH_3NH_3^+$ is larger than that of H_2O, and only a very small amount of CH_3NH_2 gas is formed in the equilibrium system, methylamine concentration can be decreased gradually through any situation causing the escape of the methylamine gas. To prevent this, the solution was kept in a tightly closed container and stored at 5°C. New stock solution was prepared every 2 weeks and the working solution was discarded after its container had been opened 6 times, to improve the consistency of the lactose standard curve.

Although Nickerson et al. (10) used sodium sulfite as the color stabilizer and recommended reading the absorbance in 10 min to obtain consistent data, the color still faded quickly. In this study, 4 tubes (one blank and 3 samples) per batch were used in the color developing procedure (65°C, 25 min) and the absorbances were read in a spectrophotometer within 2 min. The absorbance changed only at the third decimal reading when the first tube was checked again after all 4 tubes were read in the spectrophotometer; there was a difference in the second decimal reading after 5 min delay.

Figure 1 shows standard curves obtained under different conditions. Although all correlation coefficients are above 0.99, there are significant differences in the slope of the regression lines, which means they differ not only in sensitivity but also in the dimension of error caused by the sequence of the samples measured, because of unstable color.

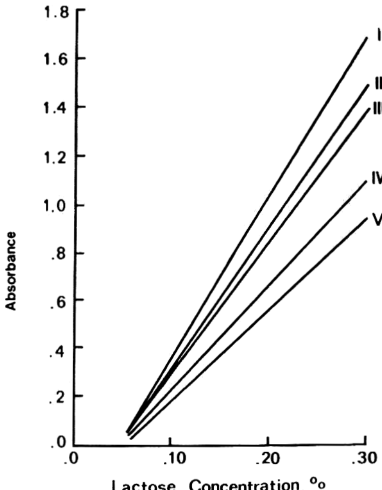

Figure 1. Standard curves under different treatment conditions.

I Dried methylamine HCl, read 4 tubes in 2 min.
II Dried methylamine HCl, read 8 tubes in 5 min.
III Dried methylamine HCl, read 15 tubes in 10 min.
IV Dried methylamine HCl, but solution was stored >3 weeks in refrigerator.
V Undried methylamine HCl.

Effect of Lactose Hydrolysis on Freezing Point Depression

Table 6 shows the regression line, correlation coefficient, and freezing point depression for each 1% lactose hydrolyzed in all substrates treated with different enzymes. From these regression lines, the degree of lactose hydrolysis ranging from 50 to 75% in respect to freezing point depression was calculated (Table 7).

Most of the lactases used in this research did coagulate milk protein at 42°C within 2 h, indicating some protease contamination in these lactases. Amino acids or other small molecules liberated through the action of protease influenced the freezing point depression measurement.

Analysis of Variance and Duncan's Multiple Range Test on Freezing Point Depression

Freezing point depression data for each 1% lactose hydrolyzed were analyzed using analysis of variance followed by Duncan's multiple range test (13). Two-way analysis of variance showed significant differences at the 5% level among fungal enzymes but not among acid substrates. Duncan's multiple range test indicated that there were significant differences in the data between enzyme D and others. Enzyme D was the only fungal lactase from *Aspergillus niger*. One-way analysis of variance and Duncan's test showed significant differences between freezing point depression data of any 2 of the 3 neutral substrates.

Summary

A direct linear relationship was indicated between freezing point depression and lactose

Table 6. Regression lines of freezing point depression (FPD) against lactose hydrolysis and FPD values for each 1% lactose hydrolyzed

Substrate	Enzyme	Regression line[a]	Correlation coeff.	FPD for each 1% lactose hydrolyzed[b]
AW	A	$Y = 421.36X + 2.27$	0.9897	0.0459
AW	B	$Y = 406.14X + 5.63$	0.9896	0.0457
AW	C	$Y = 419.87X + 6.68$	0.9804	0.0444
AW	D	$Y = 415.43X - 0.36$	0.9875	0.0477
AWC	A	$Y = 434.00X + 4.35$	0.9852	0.0436
AWC	B	$Y = 398.07X + 9.29$	0.9763	0.0447
AWC	C	$Y = 399.00X + 6.68$	0.9826	0.0461
AWC	D	$Y = 409.51X - 2.19$	0.9841	0.0497
AWP	A	$Y = 408.92X + 5.99$	0.9799	0.0480
AWP	B	$Y = 390.44X + 7.78$	0.9721	0.0487
AWP	C	$Y = 418.77X + 4.85$	0.9896	0.0466
AWP	D	$Y = 448.07X - 8.15$	0.9640	0.0501
SW	E	$Y = 431.22X - 1.55$	0.9949	0.0494
SM	E	$Y = 434.15X - 2.00$	0.9924	0.0471
LFM	E	$Y = 422.66X + 0.24$	0.9938	0.0447
Mean				0.0468
Range				0.0436–0.0501

[a] Y = FPD, X = hydrolysis, %. All data are means of duplicate or triplicate trials.
[b] FPD for each 1% lactose hydrolyzed = FPD of 90% hydrolysis calculated from regression line/lactose % in blank × 90%.

hydrolysis in various substrates hydrolyzed with lactase from several microorganisms. Conditions causing variations in the data for each 1% lactose hydrolyzed were: (a) oligosaccharides formed in the lactose hydrolysis; (b) inconsistent freezing point readings of the cryoscope at the low freezing points measured; (c) protease contamination in lactase concentrates; (d) hydration and volume of the non-protein components in the commercial enzymes; (e) unstable color complex formed by lactose in methylamine solution; and (f) difficulty in the use of methylamine solution.

The accuracy of this estimation depends on the accuracy of both the Nickerson et al. method for lactose determination and the cryoscopic method for freezing point measurement. Nickerson's method requires very careful attention to detail; if great care is taken, factors e and f can be minimized or eliminated and this method can recover 99.08 ± 2.58% of the theoretical lactose value (10). The cryoscopic method of freezing point measurement can detect 0.05% lactose addition in the range of lactose concentration of 1.0–3.5% and 0.1% addition in the 3.0–5.0% range, because the cryoscope gave precise data for freezing points

Table 7. Degree of lactose hydrolysis (%) ranging from 50 to 75% in respect to freezing point depression[a]

		Degree of lactose hydrolysis in respect to FPD, °H							
Enzyme	Substrate	0.110	0.120	0.130	0.140	0.150	0.160	0.170	0.180
A	AW	46.12	50.46	54.81	59.16	63.51	67.86	72.20	76.56
A	AWC	43.69	47.51	51.33	55.15	58.97	62.79	66.62	70.45
A	AWP	42.24	46.56	50.88	55.20	59.51	63.83	68.15	72.47
B	AW	49.88	54.12	58.36	62.59	66.83	71.07	75.30	79.54
B	AWC	52.97	56.94	60.90	64.90	68.88	72.85	76.83	80.81
B	AWP	49.14	53.27	57.40	61.53	65.66	69.79	73.92	78.05
C	AW	48.44	52.65	56.86	61.07	65.29	69.50	73.71	77.92
C	AWC	49.86	54.13	58.41	62.69	66.97	71.24	75.52	79.80
C	AWP	49.52	54.00	58.42	62.87	67.31	71.76	76.21	80.66
D	AW	51.57	55.88	60.20	64.52	68.83	73.15	77.46	81.78
D	AWC	51.73	55.77	59.80	63.84	57.88	71.91	75.95	79.98
D	AWP	49.89	54.15	58.41	62.67	66.93	71.19	75.45	79.71
E	SW	46.92	51.15	55.38	59.60	63.83	68.06	72.28	76.51
E	SM	45.28	49.64	53.99	58.35	62.71	67.06	71.42	75.78
E	LFM	46.23	50.52	54.82	59.11	63.40	67.70	71.99	76.28

[a] Calculated from regression line of middle level of enzyme dosage.

greater than −0.621°H as reported by Zarb and Hourigan. In this research, the freezing point of the sample was read 3 times successively and only the third reading was used, to improve accuracy and eliminate factor b. Factor d needs to be corrected by a modified Grimbleby's formula; this is beyond the scope of the present research. Factors a and c need further study too, but their effect can be eliminated if the estimation is based on the regression line of the same substrate treated with the same lactase.

Generally, there are 5 methods for lactose determination in hydrolyzed substrates, i.e., gas-liquid chromatographic, high pressure liquid chromatographic, enzymatic, colorimetric, and cryoscopic methods. The cryoscopic method is the simplest and quickest method among these, and it offers great accuracy and consistency.

The cryoscopic method reported by Zarb and Hourigan required 1 h incubation of the lactose-containing solution at 37°C to determine the content of lactose, while in the Baer et al. method, the degree of lactose hydrolysis was estimated in 2-3 min once the freezing point depression against hydrolysis regression line was established. This study expanded the application of the method of Baer et al. (2).

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ALCOHOLIC BEVERAGES

Westphal Plummet Determination of Alcohol in Distillates

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

A new table gives the percent alcohol by volume in distillates from density values obtained at room temperature by using a Westphal plummet.

Direct determination of the density of an alcohol–water mixture at room temperature, using a Westphal plummet, affords a rapid and convenient method for determination of alcohol content. If a table can be devised for the necessary temperature corrections, the method combines the convenience of alcohol hydrometers with the accuracy of pycnometers. The principle of such a method has existed since Archimedes; data for preparing a table were fairly well advanced by the 19th century. A necessary table has been calculated here to give accurate results for room temperatures about 20–30°C. The basic data used are wholly contained in the Bureau of Standards Circular No. 19 (1) which also contains the data needed to modify the table to cover other temperature ranges, i.e., from 15 to 25°C.

Derivation of the Method

A 2-pan analytical balance is used as a working instrument in the following derivation. A Westphal plummet is hung from the scale hook in a pan, so that it can be weighed in air or immersed in liquid. The sample container stands on a support resting on the floor of the balance and is so constructed that it does not disturb the free movement of the pan or the pan arrester.

The following terminology is adopted:

M = mass of plummet
V = volume of plummet
W_a = recorded weight of plummet in air
W_{H_2O} = recorded weight of plummet in distilled water
W_s = recorded weight of plummet in liquid sample
D_a = density of air
D_{H_2O} = density of water
D_s = density of sample
8.5 = density of brass weights

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If the plummet is immersed in the liquid sample, the following relation holds:

$$M - VD_s = W_s - (W_s/8.5)D_a$$

$$M - VD_s = W_s(1 - D_a/8.5) \quad (1)$$

For the plummet immersed in distilled water:

$$M - VD_{H_2O} = W_{H_2O}(1 - D_a/8.5) \quad (2)$$

For the plummet weighed in air:

$$M - VD_a = W_a(1 - D_a/8.5) \quad (3)$$

Subtracting Equation *2* from Equations *1* and *3*, and dividing the first result by the second:

$$D_s = D_{H_2O} - (D_{H_2O} - D_a) \times (W_s - W_{H_2O})/(W_a - W_{H_2O})$$

Dividing by the density of water:

$$D_{t/t} = 1 - (1 - D_a/D_{H_2O}) \times (W_s - W_{H_2O})/(W_a - W_{H_2O})$$

Taking the density of air as 0.00118 g/mL, the values of the term $(1 - D_a/D_{H_2O})$ are: 20°C, 0.998818; 25°C, 0.998817; and 30°C, 0.998815. Therefore, without serious error, we may assume as constant the value at 25°C so that the final operating equation is:

$$D_{t/t} = 1 - 0.998817 \times (W_s - W_{H_2O})/(W_a - W_{H_2O}) \quad (4)$$

where $D_{t/t}$ is the vacuum density of the sample relative to the vacuum density of distilled water at the same temperature.

All we have to do is to weigh the plummet immersed in the liquid sample at room temperature, record the temperature of the liquid to 0.1°C, compute $D_{t/t}$ from Equation *4*, and look up the alcohol percent from the specially prepared table. As with pycnometers, some preliminary calibration must be done, i.e., the weight of the plummet in air and its weight in distilled water at various temperatures. The weight of the plummet in water varies linearly with temperature, so that subsequently it can be found from a prepared graph or table of W_{H_2O} vs temperature. Westphal plummets are rather small, ca 5

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Table 1. Percent alcohol by volume at 60°F (15.56°C) from $D_{t/t°C}$
Temperature correction = (25 − t°C)(Factor) in % alc./vol.

$D_{t/t}$°C (vacuum)	% Alc./vol.	Factors	
		t < 25°C	t > 25°C
1.00000	0.000	0.000	0.000
0.99811	1.257	0.003	0.001
0.99627	2.510	0.003	0.004
0.99447	3.758	0.006	0.004
0.99274	5.002	0.007	0.008
0.99106	6.243	0.011	0.012
0.98945	7.479	0.016	0.017
0.98788	8.712	0.022	0.022
0.98634	9.943	0.031	0.029
0.98481	11.169	0.041	0.039
0.98330	12.393	0.054	0.047
0.98184	13.613	0.066	0.059
0.98039	14.832	0.080	0.069
0.97897	16.047	0.094	0.082
0.97758	17.259	0.110	0.095
0.97619	18.469	0.129	0.108
0.97484	19.676	0.142	0.123
0.97346	20.880	0.157	0.134
0.97207	22.081	0.170	0.147
0.97065	23.278	0.187	0.157
0.96922	24.472	0.200	0.170
0.96778	25.662	0.212	0.184
0.96630	26.849	0.226	0.192
0.96481	28.032	0.239	0.202
0.96329	29.210	0.251	0.212
0.96176	30.388	0.255	0.223
0.96018	31.555	0.261	0.228
0.95856	32.719	0.266	0.233
0.95689	33.879	0.270	0.237
0.95520	35.033	0.271	0.243
0.95345	36.181	0.275	0.244
0.95168	37.323	0.276	0.249
0.94986	38.459	0.278	0.251
0.94802	39.590	0.279	0.254
0.94613	40.716	0.281	0.254
0.94422	41.832	0.281	0.256
0.94227	42.944	0.282	0.256
0.94031	44.050	0.280	0.259
0.93830	45.149	0.279	0.258
0.93626	46.242	0.278	0.257
0.93421	47.328	0.276	0.258
0.93212	48.407	0.275	0.257
0.93001	49.480	0.274	0.256
0.92787	50.545	0.273	0.255
0.92571	51.605	0.274	0.254
0.92355	52.658	0.273	0.255
0.92137	53.705	0.272	0.255
0.91917	54.746	0.271	0.254
0.91697	55.780	0.270	0.254
0.91475	56.808	0.269	0.254
0.91252	57.830	0.266	0.253
0.91026	58.844	0.265	0.251
0.90799	59.852	0.264	0.250
0.90572	60.854	0.263	0.249
0.90343	61.850	0.262	0.249
0.90113	62.837	0.263	0.247
0.89884	63.820	0.262	0.247
0.89654	64.798	0.260	0.247
0.89423	65.768	0.258	0.246
0.89191	66.732	0.257	0.245
0.88959	67.690	0.256	0.244
0.88725	68.641	0.255	0.242
0.88491	69.586	0.253	0.242
0.88256	70.523	0.252	0.240
0.88020	71.455	0.251	0.239
0.87783	72.380	0.250	0.239
0.87547	73.299	0.248	0.237
0.87309	74.211	0.247	0.236
0.87071	75.117	0.245	0.235
0.86833	76.016	0.243	0.234
0.86593	76.909	0.241	0.232
0.86352	77.794	0.240	0.230
0.86110	78.672	0.239	0.228
0.85869	79.544	0.236	0.227
0.85626	80.410	0.235	0.225
0.85383	81.269	0.233	0.224
0.85140	82.121	0.231	0.223
0.84895	82.967	0.229	0.221
0.84650	83.805	0.226	0.219
0.84404	84.636	0.223	0.217
0.84157	85.459	0.221	0.214
0.83909	86.275	0.218	0.212
0.83659	87.083	0.216	0.210
0.83408	87.885	0.213	0.208
0.83156	88.678	0.210	0.205
0.82902	89.464	0.206	0.202
0.82646	90.240	0.202	0.198
0.82389	91.008	0.198	0.195
0.82128	91.766	0.194	0.191
0.81865	92.517	0.189	0.187
0.81600	93.254	0.185	0.183
0.81332	93.982	0.179	0.178
0.81060	94.700	0.175	0.173
0.80785	95.407	0.170	0.169
0.80507	96.103	0.165	0.164
0.80225	96.787	0.159	0.159
0.79939	97.459	0.153	0.153
0.79648	98.117	0.145	0.147
0.79349	98.759	0.139	0.140
0.79045	99.386	—	0.134
0.78736	100.000	—	0.129
0.78414	(100.625)	—	0.125

cc, and elongated, so temperature equilibrium is readily attained.

Results and Discussion

The necessary table (Table 1) was computed from density values for mixtures of alcohol and water at 20, 25, and 30°C as given in Table 1 of Bureau of Standards Circular No. 19 (1). Values of $D_{t/t}$ were computed by dividing the density values by the density of water at the top of each column. Exact values of alcohol percent, of course, are obtained from our computed table

Table 2. Check of reliability of Table 1

% Alc./vol. (60°F)	$D_{22.5/22.5}$	Table 1	Δ	$D_{27.5/27.5}$	Table 1	Δ
6.128	0.99125	6.131	+0.003	0.99118	6.124	−0.004
12.373	0.98349	12.369	−0.004	0.98319	12.365	−0.008
23.424	0.97100	23.441	+0.017	0.96998	23.429	+0.005
27.935	0.96565	27.945	−0.010	0.96427	27.935	0.000
36.280	0.95433	36.286	+0.006	0.95233	36.286	+0.006
47.315	0.93554	47.315	0.000	0.93298	47.321	+0.006
57.790	0.91405	57.799	+0.009	0.91120	57.792	+0.002
67.667	0.89120	67.665	−0.002	0.88815	67.667	0.000
76.920	0.86752	76.922	+0.002	0.86434	76.915	−0.005
85.488	0.84315	85.488	0.000	0.83986	85.490	+0.002
93.281	0.81759	93.280	−0.001	0.81425	93.279	−0.002
99.947	0.78931	99.943	−0.004	0.78601	99.944	−0.003

only at 20, 25, or 30°C, but a check of the practicability of the table was made using $D_{t/t}$ values at 22.5°C and 27.5°C. To do this, we used the original data in the work of Osborne et al. (2). They measured the thermal expansion of 12 mixtures of alcohol and water to obtain the equation:

$$D_{t/4} = D_{25/4} + \alpha(t - 25) + \beta(t - 25)^2 + \gamma(t - 25)^3$$

They give the composition by weight of the 12 mixtures and the respective values of α, β, and γ. Thus $D_{22.5/4}$ and $D_{27.5/4}$ can be calculated and changed to $D_{t/t}$, using the Chapuis values for the density of water as given in Table 32 of Bureau of Standards Circular No. 19. Alcohol percent by weight is converted to alcohol percent by volume at 15.56°C by using Table No. 5 of that circular.

Table 2 presents a test of the accuracy of Table 1 and shows that it can be used for the temperature range 20–30°C.

The whole procedure is equivalent to using a very small hydrometer, nearly perfectly calibrated, capable of high precision readings, and covering the complete alcoholic range. If a test tube is used as the "hydrometer jar," only 25 mL of an alcoholic beverage sample need be distilled for an alcohol determination.

Table 1 is a condensed alcoholimetric table valid for any intermediate temperature, fractional or otherwise, between 20 and 30°C. Its correct use, however, requires full interpolation to 3 decimal places as shown in the example below:

Determined $D_{t/t}$ = 0.97012 at 26.2°C
Interpolation (Table 1)

0.97065	23.278	0.157
0.96922	24.472	0.170
−143	+1.194	+0.013
−53	+(0.443)	+(0.005)

Uncorrected percent alcohol: 23.278 + 0.443 = 23.721

Correction factor: 0.157 + 0.005 = 0.162

Temperature correction: (25 − 26.2)(0.162) = −0.194

Corrected percent alcohol: 23.721 − 0.194 = 23.527 or 23.53% by volume.

The apparent specific gravities at 20, 25, and 30°C used to find the alcohol percent from the AOAC alcoholimetric tables (3) are actually $D_{t/t}$ values in air. They can be changed to $D_{t/t}$ (vacuum) by the relationship:

$$D_{t/t}\,(\text{vac.}) = D_{t/t}\,(\text{air})(1 - D_a/D_{H_2O}) + D_a/D_{H_2O} \quad (5)$$

If the obtained $D_{t/t}$ (vac.) is checked in Table 1, the same results will be obtained.

The method has been tried successfully during the past 2 years as an independent check on the alcohol content of beverages through the distillation of a 50 mL sample. Standard equipment accompanying a Westphal balance (i.e., the plummet and the glass cylinder) was used. Thermometers built in the plummet are not accurate enough, and the temperatures were determined to 0.1°C using precision thermometers. A support for the small glass jar was glued together from thin masonite board. It has the form of a miniature bench beneath which the pan of the balance can move freely.

With the exception of the analytical balance and the precision thermometer, all other parts,

including the plummet, can be improvised. If the procedures described in the AOAC methods for measuring the sample, distilling, and diluting to volume are followed, any laboratory can make alcohol determinations using readily available equipment.

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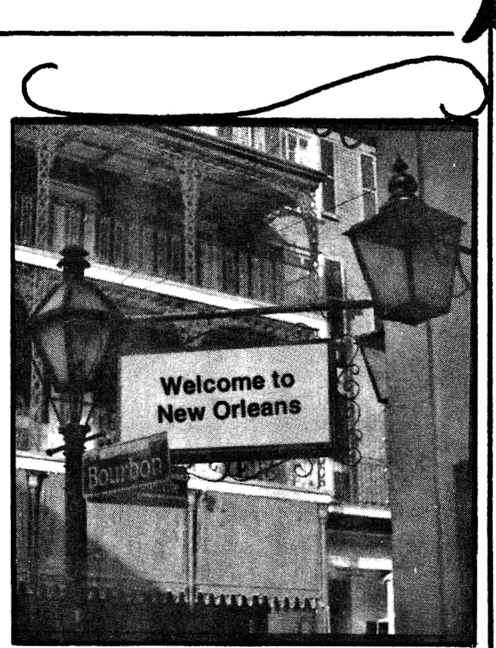
NICOLE F. HARDIN
U.S. Food & Drug Administration
4298 Elysian Fields Ave
New Orleans, LA 70122
(504/589-2471)

HERSHEL MORRIS
Louisiana Dept of Agriculture
PO Box 16390-A, University Station
Baton Rouge, LA 70893
(504/388-2755)



AOAC

Oscillating U-Tube Density Meter Determination of Alcoholic Strength: Analysis of Parameter Errors

JAN KOVÁŘ
Revenue Canada, Customs and Excise, Laboratory and Scientific Services Division, Ottawa, Ontario, Canada K1A 0L5

Determination of density by using the density meter was superior to pycnometry and hydrometry in precision, accuracy, insensitivity to operator, temperature control, speed, and adaptability to automation. The linearity between oscillation period and density in the range of ethanol-water solutions is more than adequate. The method is practically insensitive to changes in air pressure; the single most important influence on the error in results is temperature reading errors. It is recommended that the Organisation Internationale de Métrologie Légale General Formula be used to correlate densities with alcoholic strength.

The determination of density, a crucial procedure in many analyses such as the analysis of spirits for ethanol, is traditionally performed using hydrometers, pycnometers, or hydrostatic balances. The first procedure is simple but requires rather large quantities of samples if instruments of sufficient accuracy are to be used. The necessary 500–1000 mL volume may be impractical, particularly if a sample has to be pretreated such as by distillation, and, moreover, carries implicitly the increased difficulty of maintaining homogeneity and constant temperature during the measurement; the instruments must be calibrated in a procedure available only to a specialized institution. Nevertheless, this procedure is often chosen as a regulatory method because of its technical simplicity. The other 2 methods do not suffer from the drawbacks mentioned above but both require a precise volume determination of an irregularly shaped vessel (pycnometer or sinker). The procedure is available to any laboratory equipped with an analytical balance but is rather time consuming and, admittedly (1), operator-sensitive; a good deal of training is expected before reliable and accurate values can be obtained (2).

The traditional approach to density determinations is currently undergoing a revolution with the advent of density meters, instruments based on measuring the frequency of oscillation of a hollow tube filled with the sample. The frequency (or its reciprocal, the oscillation period) has a theoretically definable linear relation to density; therefore, the procedure does not require mass or volume measurement. Other published studies (2–4) agree on the superiority of density meters over hydrometry or pycnometry in respect to precision, sample amount requirements, ease of operation, temperature and homogeneity control, and speed of performance. The theoretically required linearity of the oscillation period (squared) with the density has been challenged on occasion (2,4), making the accuracy of determinations suspect unless more than 2-point calibration is made. In theory, 2 determinations exactly and sufficiently describe a calibration line for the full range; the instrument manual (5) recommends calibration on water and air as the 2 standards (points) and this is generally done. Deviations described for concentrated sugar solutions (4) could be expected because they are outside the density range limited by the 2 standards used. Deviations within the range, such as for alcohol–water solutions (3), are difficult to explain and accept. They were not experienced in a later investigation (2).

Because of our involvement in alcohol analysis we decided to investigate more closely the linearity of the period–density relationship and the influence of variations of individual parameters on the results of density meter measurements.

Experimental

Apparatus and Reagents

(a) *Density meter.*—Paar DMA 55 calculating precision density meter with manual injection.

(b) *Constant temperature bath.*—Haake F3-C circulating ultrathermostat equipped with NBS-calibrated precision thermometer, −1 to 50°C range graduated in 0.1°. Temperature of circulating liquid was also monitored in flow of water leaving cell compartment. The 2 readings coincided within limits of accuracy of temperature measurement.

(c) *Standards.*—(*1*) Distilled water or water for HPLC (Baker Analyzed), both freshly degassed by boiling; no difference in density was observed. (2) Ambient air (pressure of the day,

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Table 1. Densities and oscillation periods of samples

Sample	Nominal alcohol strength	T^{20}	ρ^{20} Density bottle	ρ^{20} Density meter
1	air	4.15746	1.200	1.20
2	95%	5.49610	811.63	811.55
3a	94%	5.49990	814.27	814.16
3b	94%	5.49991	814.27	814.17
4	60%	5.63312	907.19	907.17
5a	40%	5.60078	948.128	948.12
5b	40%	5.69082	948.128	948.12
6	40%	5.69060	947.96	942.97
7	20%	5.72654	973.68	973.69
8a	water	5.76056	998.202	998.20
8b	water	5.76052	998.202	998.20
8c	water	5.76054	998.202	998.20
8d	water	5.76053	998.202	998.20

temperature of the cell, relative humidity 30–50%). (3) Ethanol, ca 95% volume (unmatured spirits), chromatographically pure (GC); mixtures with water, strength determined by pycnometry.

(d) *Density bottles.*—Two bottles of 40 and 45 mL nominal volume, respectively, with closely fitting plastic stoppers and calibration graduations in narrow neck. Volumes were determined as average values of 7 determinations (RSD 0.011 and 0.006%, respectively). Densities of samples were determined twice in each bottle and average values were taken (RSD 0.01% or better). Pycnometers with samples are thermally equilibrated in the same Haake Ultrathermostat used for density meter, by immersing >30 min. Density in air (d^{20}) was recalculated to true densities (ρ^{20}) by standard procedure using nominal values (6) for density of air and density of weights (1.2 and 8000 kg/cu. m, respectively) as recommended by Organisation Internationale de Métrologie Légale (6):

Standardization of Meter

Performed as described in the manual (5) and elsewhere (2), using water and air as standards. Instrument constants were calculated by the equations:

$$A = (T_w^2 - T_{air}^2)/(\rho_w - \rho_{air}) \qquad (1)$$

$$B = T_{air}^2 - A \times \rho_{air} = T_w^2 - A \times \rho_w \qquad (2)$$

where T = observed oscillation periods, and ρ = tabulated densities (5) of water (w) and air (air).

Calibration was performed daily when measurements were undertaken and checked after each run by repeating density measurement of water. No calibration shifts during a run were observed. Variations of calibration constants from day to day are minor, but noticeable.

Measurement of Samples

The clean, dry cell was rinsed 2 or 3 times with sample and filled using a plastic syringe (5 mL); filling was monitored visually for absence of bubbles. After not less than 3 min, several successive readings of density were recorded; reading remained constant within 1 digit in the last place for an extended period. At the same time, readings of oscillation periods were recorded. Each sample was injected 2 or 3 times; readings of the first injection sometimes deviated slightly from the second and third, which generally agreed within 0.00001 g/cc, particularly if the prerinse was omitted. Between samples, cell was rinsed with methanol and dried with air to attain density value of air used in calibration of the day. Measurements were done predominantly at 20.00°C, with a few at 25.00°C.

Results and Discussion

Results of measurements on samples ranging from 0 to 95% volume ethanol are summarized in Table 1, showing densities of samples as determined using density bottles and meter. Corresponding oscillation periods observed are also included.

Values of densities agree well, generally within 0.01–0.02 kg/cu. m (corresponding roughly to 0.005–0.01% volume); the largest discrepancies were encountered for samples of higher strength, viz. 0.11 kg/cu. m at 95%. In this range the deviation represents only 0.03% volume, in fact, making the congruence better than that at low strengths. While the standard

Table 2. Instrument constants and corresponding densities of Sample 2 using sample and air as standards

	Density meter			Pycnometer		
Sample	A	B	ρ_{95}^{20}	A	B	ρ_{95}^{20}
1	—	—	—	—	—	—
2	15.946 988	17.265 337	811.550	15.945 414	17.265 339	811.630
3a	15.947 189	17.265 337	811.540	15.945 031	17.265 340	811.649
3b	15.947 128	17.265 337	811.543	15.945 166	17.265 339	811.643
4	15.947 070	17.265 357	811.546	15.946 718	17.265 338	811.564
5a	15.946 968	17.265 337	811.551	15.946 834	17.265 337	811.558
5b	15.947 449	17.265 337	811.527	15.947 314	17.265 337	811.533
6	15.947 331	17.265 337	811.533	15.947 500	17.265 337	811.524
7	15.947 503	17.265 337	811.524	15.947 667	17.265 336	811.516
8	15.947 388	17.265 337	811.530	15.947 388	17.265 337	811.530
9	15.946 926	17.265 337	811.553	15.946 926	17.265 337	811.553
10	15.947 157	17.265 337	811.541	15.947 157	17.265 337	811.541
11	15.947 041	17.265 337	811.547	15.947 041	17.265 337	811.547
Mean	15.947 178	17.265 330	811.540	15.946 680	17.265 338	811.500
SD	0.000 198	0.000 000	0.010	0.000 935	0.000 001	0.047

errors of the means[1] of our pycnometry determinations[2] are about 0.04–0.08 kg/cu. m, it seems reasonable to assume that most discrepancies, even so small, are due to the pycnometry determinations, with the density meter contributing only occasionally and with less than 0.04 kg/cu. m (0.005% of the value) to the overall discrepancy.

Linearity of Period-Density Relation

Although the results in Table 1, based on the assumption of a linear relationship between the differences of squared periods and the differences in densities (as expressed, e.g., by Equation *1*), themselves show a good linear relation between the 2 parameters, we decided to investigate the dependence further. To this end, the instrument constants A and B were calculated using, in turn, each of the solutions as one standard and air or water as the other, and applying Equations *1* and *2*; in each series of calculations, we used alternately the density of the sample as determined by pycnometry and by density meter. For density of water or air, the tabulated values were used (0.998202 and 0.001200, respectively).

Results of calculations are given in Tables 2 and 3, with mean values and standard deviations included. For each pair of instrument constants the density of one selected sample (No. 2), the 95% volume spirit (labeled ρ_{95}^{20}), was also calculated, using the formula of the processor; viz.

$$\rho = \frac{1}{A}(T^2 - B) \qquad (3)$$

where T is the oscillation period of that sample as shown in Table 1. The expected day-to-day variations in A and B were calculated from the 4 water and air calculation runs, giving (standard deviation included): A = 15.947 128 ± 0.000 198; B = 17.265 337 ± 0.000 000; ρ_{95}^{20} = 811.543 ± 0.000 010. The RSD in the density and in the constant A are the same, 0.0012%, reflecting changes in calibration. Density determinations made over the course of a day would therefore have no measurable variation.

When instrument constants A and B were calculated using sample and air as standards, with densities of samples determined by density meter (c.f., Table 2), the same standard deviation was obtained as for water–air calibrations alone in the whole set. This indicates no significant difference in the instrument constants and the results in the density range of alcohol–water solutions in calibrating with water–air and sample–air pairs, contrary to an earlier observation (3).

If densities determined by pycnometry are used in calculating constants and sample density, larger variations are observed (Table 2). The F values for A and ρ_{95}^{20} are 22.30 and 22.09, respectively, compared with the tabulated value of F (0.01; 12, 11) = 4.40, indicating a significant difference in variability of results. The added variability must be attributed to the single change in the calculation, i.e., to the method of density determination: Pycnometry has a larger variability than does the density meter procedure. The SD of the densities calculated in this procedure for the 95% sample (0.05 kg/cu. m) is

[1] Standard error of mean = $(SD/\sqrt{n}) \times 1.96$ (95% confidence limit).
[2] In pycnometry, deviations of 0.1 kg/cu. m are generally encountered, if recorded.

Table 3. Instrument constants and corresponding densities of Sample 2 using sample and water as standards

	Density meter			Pycnometer		
Sample	A	B	ρ_{95}^{20}	A	B	ρ_{95}^{20}
1	15.947 157	17.265 337	811.541	15.947 157	17.265 337	811.541
2	15.947 892	17.264 604	811.550	15.954 730	17.257 778	811.630
3a	15.947 018	17.265 476	811.540	15.956 555	17.255 956	811.651
3b	15.947 287	17.265 208	811.543	15.955 957	17.256 553	811.644
4	15.948 020	17.264 476	811.552	15.951 525	17.260 977	811.593
5a	15.950 722	17.261 778	811.583	15.953 271	17.259 234	811.613
5b	15.941 632	17.270 852	811.477	15.944 179	17.268 310	811.507
6	15.943 875	17.268 613	811.505	15.940 702	17.271 781	811.466
7	15.933 450	17.279 020	811.381	15.926 952	17.285 506	811.305
8a	—	—	—	—	—	—
8b	—	—	—	—	—	—
8c	—	—	—	—	—	—
8d	—	—	—	—	—	—
Mean	15.945 228	17.267 263	811.519	15.947 892	17.264 604	811.550
SD	0.005 120	0.005 111	0.060	0.009 575	0.009 558	0.112

indeed in line with the SD of pycnometric determinations on that sample (0.08 kg/cu. m).

About 100 (or more) times greater variance is observed when calculations are based on water–sample pairs, rather than water–air or sample–air pairs as is usual (Table 3). Apparently, the calibration points become too close, in terms of density as well as oscillation period, to warrant good definition of the calibration line; densities of the sample are then obtained by extrapolation rather than by interpolation, rendering the procedure inherently less accurate. A similar observation has been made with sugar solutions (4) as mentioned previously. There, also, a choice of standards so as to assure interpolation rather than extrapolation is recommended. Still, even in the worst case observed, the deviation is only about 0.1 kg/cu. m, no higher than the accepted deviation in hydrometry determinations.

The inherently lower precision of pycnometry is manifested again in this case by a higher variance in the set of values based on pycnometry densities (Table 3) compared with values based on density meter densities (Table 3).

Finally, the linearity of the period–density relation was checked by calculating the values of instrument constants A and B by linear regression (least squares method) using values in Table 1 and Equation 3 transformed to a linear form

$$y = kx + q \qquad (4)$$

where $y = \rho$, $x = T^2$, $k = 1/A$, $q = -B/A$. The results with values obtained by alternately using pycnometer and density meter densities are summarized in Table 4, with the coefficient of correlation value, r, included. An extremely high degree of correlation is indicated.[3] The values of density for the selected sample (95%, No. 2) are virtually identical with the mean values shown in Table 2, even if the constants A and B differ slightly. The differences in the constants cancel each other, as discussed below.

Table 4. Calculation of instrument constants by linear regression (values from Table 1)

	Densities determined by	
Constant	Pycnometry	Density meter
A	15.947 449	15.947 235
B	17.264 732	17.265 301
ρ_{95}^{20}	811.565	811.540
r	0.999 999 985	0.999 999 999

Influence of Variations in Constants A and B on Calculated Density

Integration of the first partial derivative of Equation 3, $\partial\rho/\partial A$, within limits of density, $\rho_2 - \rho_1 = \Delta\rho$, and $A_2 = A_1 - \Delta A$ leads to the expression

$$\Delta\rho = \frac{T^2 - B}{A^2} \times (-\Delta A) \qquad (5)$$

Solving for rounded average values, A = 16, B = 17.2, T = 5.6, gives

$$\Delta\rho = -0.055\Delta A, \quad \text{or} \qquad (6)$$

$$\Delta A = -18\Delta\rho \qquad (7)$$

[3] Statistic values for correlation are 18 000 and 100 000 compared with tabulated value of 4.437 for 99.9% confidence level, suggesting that linearity of relation is beyond any reasonable doubt.

that is, a change in density of 0.01 kg/cu. m (one unit in the last place of the meter display) is equivalent to a change in A of 0.000 18 of opposite sign. The range of T values on our instrument (and likely on other instruments of the same series) for 0–95% spirits is 5.5–5.76 and corresponds to a change of 0.000 20 to 0.000 16 in A per 0.01 kg/cu. m. Or, change in A of 0.000 10 is just barely visible in the displayed density value, corresponding to 0.005–0.006 kg/cu. m.

Similarly, integration of $\partial\rho/\partial B$ within the same limits leads to the expression

$$\Delta\rho = -\Delta B/A \qquad (8)$$

which, for the approximate value of A = 16, gives

$$16\Delta\rho = -\Delta B \qquad (9)$$

That is, a change in B exerts virtually the same influence on the resulting density as does a similar change in A, viz. 0.01 kg/cu. m corresponds to −0.000 16 in B and 0.000 10 change in B is just barely noticeable in the density reading on our instrument, corresponding to 0.0006 kg/cu. m.

Consequently, in the calculation of density by Equation *3* as performed automatically in the processor, or manually, an increase (positive error) in one constant (e.g., A) is effectively cancelled by a decrease of similar magnitude (negative error) in the other constant (e.g., B). With one constant held unchanged, the change in the other will have direct influence on the density reading predictable by Equation *7* or *9*. The relationships hold for display in specific gravity as well.

Influence of Variations in Air Density on Measurement

Substituting Equations *1* and *2* in *3* leads to the basic ratio relation

$$\frac{\rho - \rho_{air}}{\rho_w - \rho_{air}} = \frac{T^2 - T^2_{air}}{T^2_w - T^2_{air}} \qquad (10)$$

Defining the ratio of squared period differences for simplicity as K and rearranging gives, for the same value of air density at calibration and at measurement,

$$\rho = K\rho_w + (1 - K)\Delta\rho_{air} \qquad (11)$$

Integrating the partial derivative of Equation *11*, $\partial\rho/\partial\rho_{air}$, within limits ($\Delta\rho$) gives

$$\Delta\rho = (1 - K)\Delta\rho_{air} \qquad (12)$$

The value of K can be obtained from values of T in Table 1, and is equal to 0.82 for 95% alcohol and increases to 1 with strength decreasing to zero. Thereby the value of $(1 - K)$ in Equation *12* becomes 0.18 at most, and decreases to zero, and the equation can be written as

$$\text{max. } \Delta\rho = 0.18\Delta\rho_{air} \qquad (13)$$

A change in density of air is made evident in the displayed density of sample at most to 0.2 (or 20%) of its value. A change in air pressure of 10 mm Hg corresponds to about 0.015 kg/cu. m change in density of air (5) and would be manifested by a change of 0.003 kg/cu. m in the final reading at worst, and a change in atmospheric pressure of 33 mm Hg would be necessary to bring about a change in density of 1 unit in the last place of the meter display (0.01 kg/cu. m). The influence of atmospheric conditions on the result (density displayed) can be neglected.

Influence of Variations in Value of Density of Air used in Calibration

Expressing Equation *1* for 2 different values of density of air (ρ_{A1}, ρ_{A2}) as a ratio and keeping all other values constant gives

$$A_1/A_2 = (\rho_w - \rho_{A2})/(\rho_w - \rho_{A1}) \qquad (14)$$

Defining $A_1 - A_2 = \Delta A$ and $\rho_{A1} - \rho_{A2} = \Delta\rho_{air}$, the equation is simplified:

$$\Delta A/A = \Delta\rho_{air}/(\rho_w - \rho_{air}) \qquad (15)$$

Solving for rounded values of A = 16 and $\rho_w - \rho_{air} = 1$ gives

$$\Delta A = 16\Delta\rho_{air} \qquad (16)$$

A change (error) in density of air (used in the calibration) of 0.01 kg/cu. m would change the constant A by 0.000 16, which is roughly equivalent to 0.01 kg/cu. m in the displayed density reading. Or, an error in air density at the time of calibration would cause an error in density reading of about the same magnitude.

If, however, the values for air (density and oscillation period) are used to calculate the constant B, a simultaneous change in B occurs: Solving the equation derived from Equation *2*, viz.

$$\Delta B = - A\ \Delta\rho_{air} \qquad (17)$$

for A = 16 gives

$$B = - 16\ \Delta\rho_{air} \qquad (18)$$

The same error in density of air, e.g., 0.01 kg/cu. m, will cause a change in B by approximately the same amount as in A, i.e., 0.000 16, but in the

Table 5. Errors in densities due to temperature error of 0.1°, at 20°C, in kg/cu. m

Strength (% vol.)	0	10	20	30	40	50	60	70	80	90	100
True [a]	0.021	0.025	0.037	0.053	0.067	0.075	0.080	0.083	0.086	0.087	0.086
Apparent [b]	0.000	0.005	0.017	0.033	0.047	0.056	0.061	0.065	0.068	0.070	0.070

[a] Per Table II, reference 7; as observed in pycnometry or hydrometry.
[b] Calculated from true per Equation *19;* as observed with density meters.

opposite direction than in A, and change the density reading by approximately the same value in the other direction. So, the change in A, caused by an error in density of air value used in the calibration is cancelled by the simultaneous change in B, provided B is calculated using the air (second) standard values rather than the values for water or other (first) standard. If the B is not calculated using the values for air, this cancellation will not occur and an error in density is observed of about the size of the deviation in the air density, as might be derived from Equation *10* directly.

A use of the nominal value for the (average) density of air, i.e., 0.001 200 g/cc (6), for all calibrations using air as the second standard and calculating the B constant on the air values is thereby fully warranted as long as the environmental conditions do not deviate from normal (20°C, 760 mm Hg) by more than 10° or 30 mm Hg. Any difference between the true and apparent value within these limits would be less than 0.01 kg/cu. m that is beyond the resolution power of the instrument.

Influence of Temperature Reading Error

A deviation in density (error) due to difference in temperature is defined, in the first approximation, by the expansion coefficient of the sample (or difference of expansion coefficients between sample and vessel) and is, for alcohol-water mixtures, composition- and temperature-dependent. The deviations can be read from an appropriate table relating composition with density and temperature and can be also calculated quite accurately using the third term of the General Formula provided by the OIML (7). For illustration, representative values of density differences corresponding to a change in temperature from 20.00 to 20.10°C were calculated from Table II of reference 7 and are shown here in Table 5. These deviations caused by a rather slight difference in temperature of 0.1°C significantly exceed errors due to likely deviations in parameters discussed to this point. They are offset (only slightly) by minute expansions of the vessels in pycnometry or hydrometry and for small temperature differences are additive and can be applied as shown.

The density displayed on a density meter also reflects the true density of the sample at the temperature of measurement. The value displayed was calculated, however, using values of densities for the standards that were not measured but rather read from tables for the exact (supposed) temperature of measurement (e.g., 20.00°C). If the true temperature of measurement deviates from the supposed temperature, the displayed value is an apparent density (ρ^{20}_{app}) at the supposed temperature (of 20.00°C) rather than the true density at the (unknown) temperature, t (ρ^{t}_{true}). By manipulating Equations *1, 2,* and *3* it can be shown that the ratio of the apparent and true densities is equal to the ratio of densities of water (the first standard) at the 2 temperatures (supposed and true; neglecting densities of air):

$$\rho^{20}_{app} / \rho^{t}_{true} = \rho^{20}_{w} / \rho^{t}_{w} \quad (19)$$

The actual temperature of reading is inherently unknown and therefore the densities at either temperature cannot be calculated. The formula allows, however, for calculation of errors due to a definite difference in temperatures.

The apparent density thereby appears closer to the density at the supposed temperature than the true density (determined, e.g., by pycnometry) would, effectively making the error due to temperature smaller.

For 0.1°C and density readings between 800 and 1000 kg/cu. m, this shift to better values amounts to approximately 0.02 kg/cu. m. The deviations shown on the density meter are included in Table 5.

In Table 6, the values of Table 5 are transposed into corresponding strengths (% volume). The error due to temperature reading constitutes a significant single contribution to the total error in density measurement. Better and easier temperature control in density meters compared with pycnometry, let alone hydrometers, is

Table 6. Errors in strengths due to temperature error of 0.1°, at 20°C, in % volume

Strength (% vol.)	0	10	20	30	40	50	60	70	80	90	100
True[a]	0.014	0.012	0.034	0.044	0.039	0.039	0.036	0.033	0.031	0.026	0.020
Apparent[b]	0.000	0.004	0.015	0.028	0.028	0.029	0.028	0.026	0.024	0.021	0.017

[a,b] See Table 5. Values recalculated using Table IVa, reference 7.

therefore an important factor in favor of the meter.

The DMA 55 cannot directly display the alcoholic strength rather than the density without an optional circuit board (processor modifcation) because the 2 parameters are not linearly related. A 3-term formula for recalculation of specific gravities to strengths was suggested (3) with limited documentation. We feel that the extremely well experimentally founded OIML General Formula (7) would be better suitable if a circuit board (program) were to be designed, particularly for countries using the metric system of measurement.

We have developed a program numerically solving the general formula for any combination of parameters and used it to produce the tables for the Canadian System of Alcoholometry. A simplified version, for 20°C measurements only, which can be easily implemented on a relatively small programmable calculator is also available, and is used in connection with the high accuracy density determinations of the meter.

The superiority of the density meter may be expected to lead, in those applications where cost is not the deciding factor, to the replacement of hydrometry and pycnometry in practice by oscillating tube density meters.

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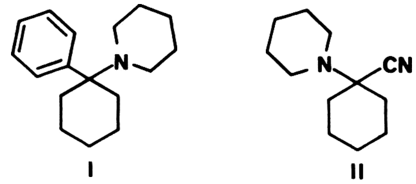
DRUGS

Liquid Chromatographic Separation of Some Alkylaminocyclohexanecarbonitriles from Phenylcyclohexylamines Related to Phencyclidine

GEORGE M. WALL[1] and C. RANDALL CLARK[2]
Auburn University, School of Pharmacy, Department of Pharmacal Sciences, Division of Medicinal Chemistry, Auburn University, AL 36849

The synthesis of five 1-aminocyclohexanecarbonitriles and the corresponding 1-phenylcyclohexylamines related to phencyclidine is reported. The compounds are separated by reverse phase liquid chromatography in an acidic methanol-water solvent system.

Phencyclidine (PCP), I, has been a popular drug of abuse in recent years. Some samples of the drug involved in adverse reactions have been shown to be contaminated with a toxic synthetic precursor, an organic cyanide, 1-piperidinocyclohexanecarbonitrile (PCC), II (1). The LD_{50} reported for PCC is 2.5 times lower than that for PCP (2). In combination, PCC effectively lowers the LD_{50} of PCP (2).



The release of hydrogen cyanide in vivo has been suggested as a possible mechanism for the toxic effects of PCC (2-4). The decomposition of α-aminonitriles such as II to yield an enamine and hydrogen cyanide occurs in the presence of heat (1, 4), moisture, and active silica gel (5). This decomposition has made some instrumental analyses of the α-aminonitriles difficult. Helisten and Shulgin (5) observed the degradation of PCC to the corresponding enamine and further decomposition to piperidine and cyclohexanone when they attempted thin layer chromatographic procedures on silica gel plates. Gas chromatographic (GC) analysis of 1-cyanocycloalkylamines has resulted in degradation (5, 6) in some cases. However, Ballinger and Marshman (1) recently reported an accurate GC procedure for determining PCC.

The similar chemical properties of the 1-phenyl-1-cyclohexylamines and the 1-aminocyclohexanecarbonitriles suggest that these compounds would not be easily separable in a synthetic mixture. Indeed, the observation of PCP samples contaminated with various amounts of PCC lends support to this assumption (1). Controls on the availability of piperidine are tightening; therefore, other amines are being used for the clandestine synthesis of arylcyclohexylamines similar to PCP. In this study, a series of 5 alkylaminocyclohexanecarbonitriles and their respective 1-phenylcyclohexylamines were synthesized and separated by liquid chromatography.

Experimental

Synthesis of Compounds

All amines and other chemical precursors were reagent grade unless otherwise specified. Alkylaminocyclohexanecarbonitriles from piperidine, pyrrolidine, morpholine, methylamine, and ethylamine were synthesized as described by Kalir et al. (7). Potassium cyanide was added to an aqueous acidic solution of cyclohexanone and amine and the resulting solution was stirred (without cooling) for at least 2 h. Products were purified by distillation and/or recrystallization.

The 1-phenylcyclohexylamines were also prepared according to Kalir et al. (7). Tertiary amine products derived from piperidine, pyrrolidine, and morpholine were prepared from the carbonitrile intermediates by treatment with a molar excess of phenylmagnesium bromide in ether solution. Secondary amine products were obtained by treating the imine formed from cyclohexanone and the primary amine (methyl or ethylamine) with a molar excess of phenyllithium in ether solution.

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[1] Present address: University of Mississippi, School of Pharmacy, Department of Medicinal Chemistry, University, MS 38677.
[2] Address correspondence to this author.

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Figure 1. Common synthetic method for the production of PCP-type compounds.

Elemental analyses (C, H, N), infrared, and nuclear magnetic resonance data were consistent with the assigned structures. Hydrochloride salts of all products were obtained by precipitation from ethereal solution with HCl gas.

Procedure

Liquid chromatograph consisted of Waters Model 6000A pump, Model U6K injector with 2 mL loop, and Hitachi Model 100-60 spectrophotometer equipped with 20 μL flow cell. Separations were accomplished by using a 25 cm × 4.6 mm id Partisil-ODS analytical column preceded by a 7 cm × 2.1 mm id guard column dry-packed with Whatman Co: Pell ODS. Ultraviolet (UV) spectrophotometer was operated at 215 nm and 0.02 AUFS. Aqueous portion of mobile phase was pH 2.96 buffer prepared by mixing 0.1M KH_2PO_4 and 0.1M K_2HPO_4 (9 + 1). Chromatographic mobile phase consisted of 55% buffer and 45% methanol (HPLC grade) at flow rate of 2.0 mL/min. Buffer was prepared in double distilled water and chromatographic separations were performed at ambient temperature.

Results and Discussion

PCP-type drugs of abuse can be produced from available laboratory chemicals in a 2-step reaction (Figure 1). The initial synthetic product is an aminocyclohexanecarbonitrile such as PCC. The chemical properties of the intermediate nitrile and the final product are very similar. Both the nitrile and the phenylcyclohexylamine are organic bases that form crystalline hydrochloride salts. Thus, unreacted nitrile easily contaminates the isolated arylcyclohexylamine. Previous work (2) has shown that PCC is more toxic than PCP and that at least part of the PCC toxicity may be attributed to cyanide poisoning.

The structures of the analogs of PCC and PCP included in this study are shown in Figures 2–6. The isolation of the final products free from the nitrile intermediate required careful control of the reaction conditions and repeated extractions during workup. Controls on the availability of piperidine are tightening and other amines are being used for the clandestine synthesis of arylcyclohexylamines similar to PCP. It seems

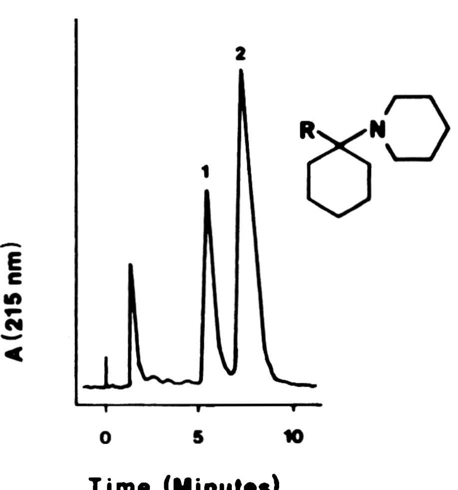


Figure 2. Chromatographic separation of the aminocyclohexanecarbonitrile and phenylcyclohexylamine prepared from piperidine. Peak 1, R = CN; peak 2, R = C_6H_5. Chromatographic conditions as described in text.

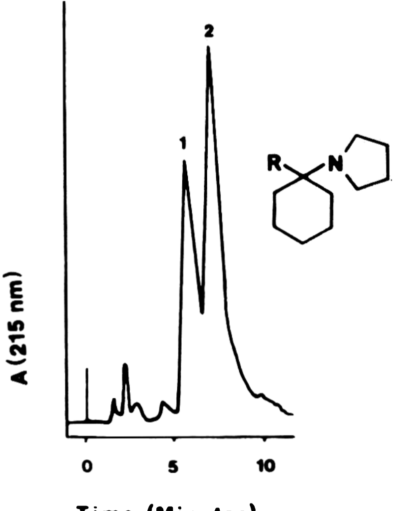


Figure 3. Chromatographic separation of the aminocyclohexanecarbonitrile and phenylcyclohexylamine prepared from pyrrolidine. Peak 1, R = CN; peak 2, R = C_6H_5. Chromatographic conditions as described in text.

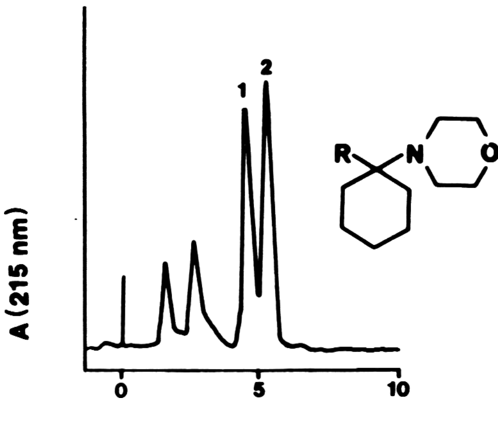


Figure 4. Chromatographic separation of the aminocyclohexanecarbonitrile and phenylcyclohexylamine prepared from morpholine. Peak 1, R = CN; peak 2, R = C_6H_5. Chromatographic conditions as described in text.

reasonable to assume that these PCP analogs will be prepared by the reaction shown in Figure 1, and contaminated with their respective nitrile precursors, as PCP is contaminated with PCC. Studies on the lethality of the intermediate nitriles in this laboratory have shown LD_{50} values similar to that of PCC.

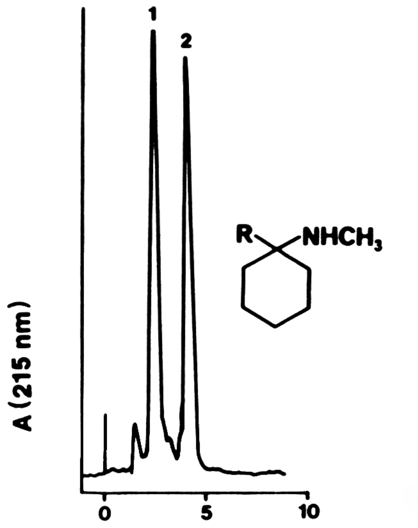
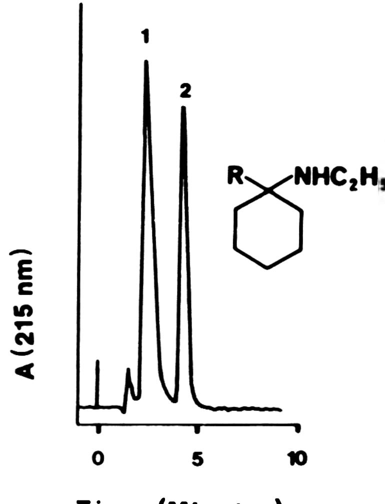


Figure 5. Chromatographic separation of the aminocyclohexanecarbonitrile and phenylcyclohexylamine prepared from methylamine. Peak 1, R = CN; peak 2, R = C_6H_5. Chromatographic conditions as described in text.



Figue 6. Chromatographic separation of the aminocyclohexanecarbonitrile and phenylcyclohexylamine prepared from ethylamine. Peak 1, R = CN; peak 2, R = C_6H_5. Chromatographic conditions as described in text.

In a recent study, Soine et al. (4) found that 33% of PCP samples confiscated in one area were contaminated with PCC. Other work (2) has shown some seized street samples to contain PCC amounts 3–4 times that of PCP and a few samples to contain only PCC. Because the chemical properties of intermediate nitrile and final product are very similar, even an unsuccessful Grignard-type reaction (second reaction in Figure 1) results in the isolation of a crystalline hydrochloride salt. The crystalline product could be the nitrile, arylamine, or, most likely, a mixture of the two.

The reverse phase liquid chromatographic separations of the 1-aminocyclohexanecarbonitriles from the corresponding 1-phenylcyclohexylamines are shown in Figures 2–6. The detectability of these compounds is very poor at the standard wavelength (254 nm) used to monitor liquid chromatographic effluent. This is particularly true for the aminocyclohexanecarbonitriles. These compounds have a wavelength of maximum absorption in the 215 nm range and are essentially transparent at higher wavelengths. Thus, for maximum sensitivity, the UV detector was operated at 215 nm. Even at this wavelength the nitriles are not particularly strong chromophores.

Compounds were separated on a C_{18} reverse phase column using an acidic methanol–water mobile phase. The aqueous buffer was pH 2.96

and this acidic medium should be sufficient to shift the equilibrium between protonated amine and its corresponding free base heavily toward the protonated amine. Thus, the species being chromatographed is the protonated amine or the hydrophilic protonated amine phosphate ion-pair. Attempts at separations at higher pH levels resulted in poor resolution and peak shape, especially for the aminocyclohexanecarbonitriles. This may indicate that the nitrile compounds are undergoing decomposition reactions during the separation process under neutral or basic conditions. The formation of hydrophobic ion-pairs by adding 1-heptanesulfonic acid to the mobile phase did not enhance resolution. Normal phase separations were equally unsuccessful. The chromatograms in Figures 2-6 show that the nitrile precursor elutes ahead of the arylcyclohexylamine in this reverse phase system. Resolution was adequate in these examples; however, peak tailing remains a problem in some cases. Detection limits were in the range of 1×10^{-7} mole/injection for the nitriles and arylcyclohexylamines. An injection of this amount of sample produced a peak of 5:1 signal-to-noise ratio or greater for all compounds studied.

The results of this work indicate that liquid chromatography is a useful technique for the separation of 1-aminocyclohexanecarbonitriles from the corresponding 1-arylcyclohexylamines and that maximum sensitivity by UV spectrophotometry requires monitoring at 215 nm. This procedure allows for the analysis of mixtures of 1-aminocyclohexanecarbonitriles in the presence of the corresponding arylcyclohexylamine.

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Protein Nitrogen Unit Precipitation Procedure for Allergenic Extracts: Collaborative Study

JOAN C. MAY and JENNY T. C. SIH
Food and Drug Administration, Bureau of Biologics, 8800 Rockville Pike, Rockville, MD 20205

Collaborators: Allergy Laboratories, J. Best, G. Douglas, J. M. Rancour, H. R. Renker, F. Spingola, L. Van Daele, and S. Zudeck

Protein nitrogen unit (PNU) determination is one of the methods used to test and label the concentration of allergenic extracts. This recently standardized method is applicable to all allergenic extracts. One PNU/mL is equivalent to 1×10^{-5} mg nitrogen determined to be in the material precipitated from 1 mL allergenic extract by phosphotungstic acid (PTA), a protein precipitant. The nitrogen is quantitated by the Kjeldahl method or another analytical method of equivalent accuracy and precision. A collaborative study of the optimized PNU precipitation method in which 6 samples were analyzed in duplicate by 6 laboratories using the Kjeldahl method for the determination of nitrogen yielded a mean of 0.1358 mg N/mL, a repeatability standard deviation and coefficient of variation of 0.0071 mg N/mL and 5.23%, respectively, and a reproducibility standard deviation and coefficient of variation of 0.0188 mg N/mL and 13.84%, respectively. The method has been adopted official first action.

The methodology for the protein nitrogen unit (PNU) precipitation has been standardized recently (1) by establishing optimum conditions for protein precipitation in allergenic extracts by phosphotungstic acid (PTA). PTA is preferred over other protein precipitants for allergenic extracts because it appears to be more specific for the precipitation of allergenically active material (2). This paper describes a collaborative study of the optimized precipitation method. The PNU methodology is one of the procedures (3) used to test and label the concentration of allergenic extracts; it is applicable to all allergenic extracts. One PNU/mL is equivalent to 1×10^{-5} mg nitrogen (10 ng N) determined to be in the material precipitated from 1 mL allergenic extract by phosphotungstic acid (PTA), a protein precipitant. The nitrogen is quantitated by the Kjeldahl method or another analytical method of equivalent accuracy and precision. The introduction of this optimized PNU methodology will improve the standardization of licensed allergenic extracts with respect to their concentration and labeling, which heretofore has been inconsistent. A consistently determined and labeled PNU value will aid the physician in assessing the equivalence of competitive extracts and in preparing a standardized patient dose.

Other units and methodologies for the chemical standardization of allergens are in use. However, those methods in current use and those potentially applicable are fraught with inaccuracies in practice (3). These include the Noon-unit (1 g pollen is equivalent to 10^6 Noon-units) and expressing extract potency on *weight to volume* basis (grams of dry allergenic material per volume of extracting solvent). Standardization of allergenic extracts by their amino acid composition has not been feasible, because acid hydrolysis in the presence of carbohydrate-rich allergens leads to heavy losses of amino acids and poor recoveries. The possibilities for standardization of allergenic extracts by taking advantage of absorption or fluorescence spectra have not yet been fully explored. However, because allergenic extracts usually contain nonallergenic proteins with similar physicochemical properties, these proposed methods are not likely to be highly specific for allergenically active proteins.

The PNU precipitation method for allergenic extracts was collaboratively studied as described below.

Collaborative Study

The collaborators were provided with samples, a copy of the PTA precipitation method, and sample preparation information. For consistency in the evaluation of the PTA precipitation method, collaborators were requested to determine the nitrogen in the precipitate by the Kjeldahl method of Kabat and Mayer (4) with methyl red-bromocresol green (5) as the titration end point indicator. The details of this Kjeldahl method were provided. Two laboratories volunteered to follow the prescribed PNU precipi-

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The recommendation of the Associate Referee was approved by the General Referee and Committee B and adopted by the Association. See *J. Assoc. Off. Anal. Chem.* 65, March issue (1982) for detailed reports.

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tation but to determine the nitrogen content in the precipitate by AutoAnalyzer methods. It is valuable to have such comparative data, because some allergenic manufacturers use AutoAnalyzer methodology in their PNU tests. However, because this collaborative study is limited to results obtained by the Kjeldahl method and 2 laboratories do not provide enough data to yield valid statistical conclusions about the AutoAnalyzer method itself, the AutoAnalyzer results are included here for information only.

For the collaborative study, 10 samples were sent to 9 laboratories to be analyzed in duplicate. The collaborative study design was that of Steiner (6); the statistical analysis was aimed at the calculation of the errors associated with the reproducibility and repeatability of the PNU precipitation. The sample labeled PNU 4 was Bureau of Biologics Nitrogen Reference No. 3, a solution of bovine albumin in water. The other samples were various types of allergenic extracts as listed below:

PNU 1—Alum-precipitated *Alternaria tenuis* extract
PNU 2—Glycerinated honey bee extract
PNU 3—Alum-precipitated house dust extract
PNU 5—Freeze-dried BoB Reference Short Ragweed Pollen Extract No. 1
PNU 6—Aqueous *Alternaria* extract
PNU 7—Aqueous *Alternaria* extract
PNU 8—Aqueous *Alternaria* extract
PNU 9—Glycerinated *Alternaria* extract
PNU 10—Aqueous *Alternaria* extract

The allergenic extracts used in this study were purchased from various U.S. manufacturers. The extracts were suitable for release and clinical use.

A practice sample was distributed with the collaborative samples. The collaborators were given the nitrogen content of this sample so that they could validate their PNU precipitation and their method for the determination of nitrogen. The practice sample was the National Bureau of Standards (NBS) Standard Reference Material (SRM) No. 927 Bovine Serum Albumin—7% Solution (Department of Commerce, Washington, DC).

Allergenic Extracts—Protein Nitrogen Unit Precipitation Method Official First Action

Principle

Protein is pptd from allergenic ext by phosphotungstic acid, and N in ppt is detd by appropriate Kjeldahl procedure. Protein nitrogen unit (PNU) is equiv. to 1×10^{-5} mg N.

Reagent

Phosphotungstic acid (PTA) precipitating solution.—Dissolve 15.0 g PTA in ca 70 mL H_2O. Add 22.2 mL HCl and dil. to 100 mL with H_2O.

Determination

Combine vol. of allergenic ext indicated below with 0.25 mL HCl in 12 mL conical centrf. tube. Use 2 mL sample when approx. PNU value of ext is not known. When approx. PNU value of ext is known, analyze following vols:

Allergenic ext, PNU/mL	*Vol., mL*
>35 500	1
15 500–35 500	2
<15 500	3

Add 1 mL PTA pptg soln. Mix thoroly. Let stand 1 h at room temp. (22 ± 3°).

Centrf. mixt. at room temp. at 2700 rpm (rotor radius = 10.80 cm) for 10–15 min (rel. centrifugal force measured to tip of sample tube = g = 880).

Test for completeness of pptn by adding 5 drops PTA soln. Check visually for turbidity in supernate. If turbidity develops, add addnl 0.5 mL PTA soln. Let mixt. stand 1 h at room temp. Recentrf. at 2700 rpm for 10–15 min (room temp.).

Poor off supernate. Invert centrf. tube to drain ppt. Do *not* wash ppt.

Dissolve ppt in 10 mL 2% NaOH by first adding 3 mL 2% NaOH with vol. pipet. Use vortex mixer to loosen ppt. Add 7 mL 2% NaOH (vol. pipet). Mix thoroly. Det. N by appropriate Kjeldahl method. PNU/mL = $10^5 \times$ mg N/mL.

Nitrogen Determination

Reagents

(a) *Sulfuric acid.*—Sp. gr. 1.84, N-free.

(b) *Copper sulfate.*—$CuSO_4.5H_2O$, N-free. Prepare saturated aqueous solution.

(c) *Acid solution.*—Add ca 40 mL saturated aqueous $CuSO_4$ to 9 lb bottle of H_2SO_4 in 10 mL portions with thorough mixing. After several days, excess anhydrous $CuSO_4$ crystallizes and the supernatant acid is ready to use.

(d) *Potassium sulfate.*—N-free.

(e) *Sodium hydroxide solution.*—50% NaOH.

(f) *Boric acid solution.*—2%.

(g) *Indicator solution.*—Methyl red-bromocresol green solution. Mix 1 part 0.1% alcoholic

methyl red solution with 5 parts 0.1% alcoholic bromocresol green solution.

(**h**) *Hydrochloric acid.*—0.01N. Prepare as in **50.011** and standardize as in **50.015** or **50.017,** or use 0.01000N standard HCl (purchased as standard).

Apparatus

See **47.022.**

Determination

Pipet 9 mL prepared sample into 30 mL digestion flask. Add ca 500 mg K_2SO_4, 3 boiling stones, and 2 mL $CuSO_4$-H_2SO_4 solution. Place flask in digestion rack. Heat carefully and digest sample until solution turns colorless. Continue digestion for additional ½ h. Cool and place thin film of petroleum jelly on rim of flask. Transfer digest and boiling chips to distillation apparatus and rinse flask 5 or 6 times with 1–2 mL portions of water. Place 125 mL Erlenmeyer flask containing 5 mL 2% H_3BO_3 solution and 5 drops of indicator under condenser with tip extending below surface of solution. Add ca 6 mL NaOH (50% w/w) to still. If distillation apparatus uses steam distillation, distill at rate of 5 mL/min and collect about 50 mL. If apparatus does not introduce steam into distilling flask, collect 10–15 mL distillate and dilute to ca 50 mL with water. Titrate distillate with 0.1N HCl to end point (pinkish purple). Perform blank determination in same manner, using water in place of sample.

Calculations

mg N/mL = [(mL HCl − mL HCl blank) × normality × 14.007 × 10/9]/mL sample

Results and Discussion

Useful results were obtained from 6 of the 7 laboratories that used the Kjeldahl method for the determination of nitrogen. The seventh laboratory (Laboratory G) reported results that differed greatly from the other collaborators' results. The results for 3 of this laboratory's samples were outliers by Dixon's test (7). Laboratory G's results were not included in the data analysis.

Two laboratories (Laboratories H and I) used AutoAnalyzer methods (not the Kjeldahl nitrogen method) to determine the nitrogen content of the PTA precipitate. The results for these 2 laboratories were not included in the statistical analysis of the data with those of the 6 laboratories that used the Kjeldahl nitrogen method.

Of the original 10 samples procured for this collaborative study, 2 samples (PNU 2 and PNU 9) were found after analysis to be below the detection limit for this method. These samples were eliminated from the collaborative study. Originally PNU 2, PNU 9, and PNU 6 were ordered with the intent of including samples in the collaborative study which were situated at the lower end of the analytical range encountered. Of these 3 samples, only PNU 6 had an acceptable PNU level. Two other samples (PNU 5 and PNU 7) were also eliminated from the study because several laboratories deviated from the prescribed PNU collaborative precipitation in their treatment of these samples.

Table 1 lists the collaborative results. For convenience, the units mg N/mL are used (1 PNU/mL = 1×10^{-5} mg N/mL).

PNU 4 was an aqueous solution of bovine albumin (BoB Nitrogen Reference No. 3) that has been used as a control sample for PNU determinations at the Bureau of Biologics. The average value of 109 Kjeldahl determinations on the PTA precipitate of BoB Nitrogen Reference No. 3 was 0.149 ± 0.005 mg N/mL with a relative standard deviation of 3.6%. The mean of the 12 collaborative results for PNU 4 was 0.143 ± 0.011 mg N/mL with a relative standard deviation of 7.4%. The relative error of this result from the BoB average was 4.03%. In terms of recovery, the average of the results of the 6 collaborators for this sample was 96% of the known mg N/mL or PNU/mL value. This is an indication of adequate accuracy and precision on the part of the collaborators. The error inherent in the Kjeldahl nitrogen method is usually quoted as ±0.01 mg N/mL (4). The error associated with the PTA precipitation has not been assigned a value. The collaborators' average value was 0.006 mg N/mL lower than the BoB average value for PNU 4. This error is well within the ±0.01 mg N/mL usually associated with the Kjeldahl nitrogen determination.

The valid data in Table 1 were examined statistically by using the method of Steiner (6). The resulting statistical data for repeatability (variation within laboratory) and reproducibility (variation among laboratories) are considered to be acceptable: mean, 0.1358 mg N/mL; repeatability SD, 0.0071 mg N/mL; reproducibility SD, 0.0188 mg N/mL; repeatability CV, 5.23%; reproducibility CV, 13.84% (results based on 72 determinations by 6 laboratories: Collaborators A, B, C, D, E, and F). The repeatability coefficient of variation is less than 10%. The reproducibility coefficient of variation is greater than 10% (13.84%), which is considered adequate be-

Table 1. Collaborator results for the determination of PNUs in allergenic extracts (1 PNU/mL = 1 × 10^{-5} mg N/mL)

	Sample, mg N/mL					
Coll.[a,b,c]	PNU 1	PNU 3	PNU 4	PNU 6	PNU 8	PNU 10
A	0.109	0.176	0.142	0.019	0.168	0.181
	0.113	0.180	0.145	0.018	0.165	0.159
B	0.103	0.149	0.133	0.030	0.144	0.155
	0.100	0.155	0.133	0.015	0.144	0.155
C	0.117	0.175	0.145	0.016	0.175	0.183
	0.117	0.171	0.148	0.023	0.206	0.183
D	0.105	0.179	0.142	0.028	0.182	0.184
	0.104	0.182	0.148	0.023	0.172	0.182
E	0.131	0.201	0.168	0.037	0.197	0.184
	0.146	0.205	0.148	0.054	0.193	0.205
F	0.100	0.159	0.129	0.032	0.162	0.243
	0.098	0.151	0.132	0.033	0.155	0.235
G	0.133[d]	0.178[d]	0.189[d]	0.004[e]	0.360[e]	0.062[e]
	0.134	0.178	0.189	0.003	0.358	0.061
H	0.130[d]	0.170[d]	0.140[d]	0.020[d]	0.150[d]	0.170[d]
	0.120	0.170	0.140	0.020	0.170	0.150
I	0.109[d]	0.183[d]	0.142[d]	0.017[d]	—	—
	0.109	0.173	0.140	0.012	—	—
Mean	0.1119	0.1736	0.1428	0.027	0.1719	0.1874

[a] Duplicate pairs.
[b] Collaborators A, B, C, D, E, F, and G used the Kjeldahl method to determine the nitrogen content of the PTA precipitate.
[c] Collaborators H and I used AutoAnalyzer methods to determine the nitrogen content of the PTA precipitate.
[d] Deleted from analysis.
[e] Outlier by Dixon's test. Deleted from analysis.

cause the sample types chosen were those most difficult to assay by this method (1), for example, aqueous *Alternaria* allergenic extract.

Limited data from 2 laboratories that determined nitrogen in the PNU method by AutoAnalyzer methods indicated that, although there were insufficient data to be considered as valid collaborative evidence, this AutoAnalyzer method could be the subject of a successful future collaborative study. The results are in good agreement with the results obtained by the Kjeldahl method.

Recommendations

On the basis of the results reported here it is recommended that the protein nitrogen unit precipitation method be adopted official first action for the determination of the PNU concentration of allergenic extracts.

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Polarographic Analytical Study of Oxyphenbutazone

MOUSTAFA M. ELLAITHY, M. FAYEZ EL-TARRAS, and SAWSAN M. AMER
Cairo University, Analytical Chemistry Department, Faculty of Pharmacy, Cairo, Egypt

The polarographic behavior of the widely used anti-inflammatory agent, oxyphenbutazone, was studied. It is determined polarographically by conversion to the nitroso derivative characterized by a cathodic, irreversible, diffusion-controlled wave. The method is applied to the determination of 2.5-10 mg/100 mL of oxyphenbutazone, with an accuracy of 99.9 ± 1.38%. By differential pulse polarographic analysis, as little as 10 ppm oxyphenbutazone can be determined with an accuracy of 99.70 ± 0.99% in pure powder and in some pharmaceutical formulations.

Only limited polarographic analytical studies have been reported for the widely used anti-inflammatory drug oxyphenbutazone, 4-butyl-2-(4-hydroxyphenyl) 1-phenylpyrazolidine-3,5-dione monohydrate. The *British Pharmacopoeia* (1) identification test for oxyphenbutazone has been adapted to the determination of oxyphenbutazone and phenylbutazone by differential pulse polarography (2), but the procedure does not distinguish these 2 compounds. Adam and Joslin (3) studied the electrochemical oxidation of pyrazolone derivatives in alkaline medium, and others studied the oscillopolarographic behavior of phenazone and phenylbutazone (4).

Pelinard et al. (5) studied the electrochemical behavior of the anti-inflammatory derivatives of pyrazolones. They reported that pyrazolidine-3,5-dione and other pyrazolone derivatives, having a labile hydrogen atom at the 4-position, show some electrochemical activity in a neutral medium composed of 0.2M lithium perchlorate in ethanol, while phenazone derivatives show activity only after diazotization. We report here the further study of the polarographic behavior, together with a differential pulse polarographic determination of oxyphenbutazone.

Experimental

Apparatus

Current potential curves were recorded using a Radelkis polarograph OH-102. A Model 384-1 polarographic analyzer system (EG & G Princeton Applied Research) was used for recording differential pulse polarographic curves.

The solution to be investigated was placed either in a usual Kalousek cell or, where the solution was highly colored, in a Heyrovsky vessel with a separate reference saturated calomel electrode.

The rate of mercury flow through the capillary used for DC polarographic measurements, at a mercury height of 45 cm, was 1.8 mg/s and the drop time was 3.0 s (in distilled water). Except when the effect of mercury height was studied, a 45 cm mercury head was maintained.

A dropping mercury electrode assembly, Model 303 SMDE (Princeton Applied Research), was used for differential pulse polarographic measurements.

Reagents

(a) *Oxyphenbutazone stock standard solution.*—1 mg/mL. Prepare in ethanol.

(b) *Working standards for direct polarographic measurements.*—Prepare by suitable dilution with appropriate supporting electrolytes, such as HCl, KCl, LiCl, and NaOH solutions.

(c) *For indirect DC polarographic determinations.*—Mix 25 mL (equivalent to 25 mg) ethanolic stock standard oxyphenbutazone solution with 25 mL 1N HCl in 100 mL volumetric flask. Add 25 mL 1N sodium nitrite solution and mix thoroughly. Let reaction proceed for 10 min with occasional shaking. Stop reaction by diluting to mark with 20% KOH solution. Dilute various aliquots of obtained solution with 15 mL water–ethanol (1 + 1) mixture to obtain desired different concentrations.

(d) *For differential pulse polarographic determination.*—Dilute 10 mL (equivalent to 10 mg) ethanolic stock standard oxyphenbutazone solution to 50 mL with water. Mix 25 mL of obtained solution with 25 mL 1N HCl in 100 mL volumetric flask. Add 25 mL 1N sodium nitrite solution and mix thoroughly. Let reaction proceed as above. Before polarographic measurements, remove oxygen from solution by bubbling stream of pure nitrogen through ca 3 min.

Procedure

(a) *Tablets.*—Use 20 tablets to calculate average weight of each. Accurately weigh an amount of tablet powder containing ca 100 mg oxyphen-

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Table 1. DC polarographic determination in alkaline medium of oxyphenbutazone product with nitrous acid

Taken, mg/100 mL	Found, mg/100 mL	Recovery, %
2.50	2.45	98.0
4.17	4.10	98.3
5.00	5.10	102.0
5.83	5.80	99.5
7.50	7.60	101.3
8.33	8.30	99.6
10.83	10.90	100.6
	Mean ($P = 0.05$)	99.9
		±1.38
	SD	1.49

Table 2. Differential pulse polarographic determination of oxyphenbutazone standard by proposed procedure

Taken, ppm	Found, ppm	Recovery, %
10	9.97	99.7
20	19.89	99.5
30	30.14	100.5
40	39.60	98.8
50	50.55	101.1
100	98.7	98.7
	Mean ($P = 0.05$)	99.7
		±0.99
	SD	0.94

butazone into 100 mL volumetric flask, and extract with and dilute to volume with ethanol. Pipet 25 mL clear supernate into 100 mL volumetric flask and treat as standard solution, beginning "Add 25 mL sodium nitrite solution . . ."

(**b**) *Capsules.*—Use contents of 10 capsules to calculate average weight of each. Accurately weigh an amount of mixed contents equivalent to ca 100 mg oxyphenbutazone into 100 mL volumetric flask, and extract and dilute to volume with ethanol. Pipet 25 mL clear supernate into 100 mL volumetric flask and proceed as in (a).

(**c**) *Suppositories.*—Allow 5 suppositories to melt by gentle warming in 50°C water bath. Mix thoroughly, let solidify, weigh, and calculate average weight of each. Accurately weigh an amount of mixed suppositories equivalent to ca 100 mg oxyphenbutazone into 100 mL volumetric flask, and extract and dilute to volume with ethanol. Transfer 25 mL clear supernate to 100 mL volumetric flask and proceed as in (a).

Results and Discussion

Oxyphenbutazone has been proved to be polarographically inactive under our experimental conditions and in different supporting electrolytes. An uncertain, ill-defined reduction wave was reported (5) only in an ethanolic neutral solution of 0.2M lithium perchlorate. Attempts to improve the wave for quantitative measurements, by using different solvents and supporting electrolytes, were unsuccessful.

Oxyphenbutazone, however, being a phenolic compound with a vacant ortho position, was expected to convert to a polarographically active nitroso derivative when treated with nitrous acid (6). The most reproducible, well defined cathodic wave was obtained when the reaction between oxyphenbutazone and nitrous acid was stopped by the addition of 20% KOH solution, and the polarogram was recorded in the alkaline KOH solution as a supporting electrolyte.

Stopping the reaction with sulfamic acid (to decompose the excess nitrite in the solution), or using acidic or neutral Britton-Robinson buffer as a supporting electrolyte gave ill-defined waves.

The single cathodic wave of oxyphenbutazone obtained in distinctly alkaline solution, after treatment with nitrous acid, showed an $E_{1/2}$ of about −0.92 V vs SCE.

Effect of mercury height (h) on current (i) was studied. A linear relationship was obtained between recorded current and square root of mercury height. The log i–log h plot also shows a linear relationship, with a slope near 0.5, indicating the diffusion-controlled character of the wave. The obtained current was directly proportional to the concentration of the depolarizer over a wide range of concentration. Thus, this reduction diffusion-controlled wave is suitable for quantitative polarographic determination of oxyphenbutazone.

The logarithmic analysis plot of the wave obtained under these experimental conditions shows a linear relationship, with an exceptionally high slope value significantly different from the theoretical value corresponding to reversible system, 59 mV for a fast electrochemical monoelectronic reaction. The electrode reaction is, therefore, irreversible.

The suggested derivatization procedure for oxyphenbutazone has been applied to different concentrations (Table 1). The suggested polarographic method can determine 2.5–10.83 mg oxyphenbutazone/100 mL after derivatization, with an accuracy of 99.9% and a confidence limit of ±1.38%.

The highly sensitive differential pulse polarographic (DPP) technique has been used for the determination of oxyphenbutazone through the obtained peak current, using the suggested

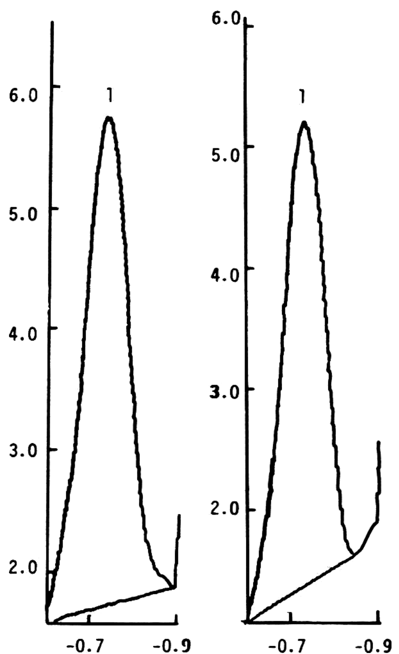

Figure 1. Differential pulse polarograms of oxyphenbutazone sample. Current, × 10^2 NA; initial E, −0.7 V; final E, −1.0 V; peak 1, −0.826 V; left, 4.100E2 NA, 50.55 ppm; right, 3.893E2 NA, 48.03 ppm.

derivatization method applied to oxyphenbutazone standards. The results obtained for 10–100 ppm oxyphenbutazone are shown in Table 2 and Figure 1.

Results for tablets, suppositories, and capsules, which are simply extracted with ethanol and then treated as standards, are given in Table 3, compared with the *British Pharmacopoeia* (1973) method.

With DPP, much smaller quantities of oxyphenbutazone, 10–100 ppm, can be determined, with an accuracy of 99.7 ± 0.99%. Tablet, capsule, and suppository excipients did not interfere, and simple dissolution in ethanol was sufficient. The accuracy of the method for pharmaceutical formulations is equally as good.

The reaction product of this derivatization reaction, under the experimental conditions, is probably due to the phenolic nature of oxyphenbutazone which, having an unoccupied ortho position, reacts with nitrous acid to give the *o*-nitroso derivative (7, 8).

Table 3. Differential pulse polarographic determination of oxyphenbutazone in some pharmaceutical preparations by the proposed method

Preparation and batch No.	Found, %	
	DDP	BP
Tandril tab., Swiss-Pharm, 091B	99.7	98.8
Oxysone tab., Nile Co., 3795	89.2	88.9
Rhumaxin tab., Alex Co., 5	100.9	101.0
Rhumaxin supp., Alex Co., 406006	98.6	101.0
Realine caps., Swiss-Pharm., 056800	100.2	98.6
Calculated Student's *t*:	0.0637	
Tabulated *t*:	2.306	
$P = 0.05$		

Feigl (9) reported that *o*-nitroso derivative, as the tautomeric oxime form, can yield metal chelates, and this has been experimentally proven to be the case with oxyphenbutazone (8). This is not the case with phenylbutazone, 4-butyl-1,2-diphenyl-pyrozolidine-3,5-dione, which was polarographically inactive either as such or after treatment with nitrous acid. Thus, phenylbutazone does not interfere in the assay for oxyphenbutazone.

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Colorimetric Determination of Certain Phenol Derivatives in Pharmaceutical Preparations

SAYED M. HASSAN, MOHAMMED I. WALASH, SANAA M. EL-SAYED, and ABDEL MALEK ABOU OUF
Mansoura University, Pharmaceutical Chemistry Department, Faculty of Pharmacy, Mansoura, Egypt

Simple colorimetric methods are reported for determining both acetaminophen and oxyphenbutazone. These methods are based on coupling between the phenolic compound and the diazonium salts of both sulfanilic acid and *p*-nitroaniline; the optimum conditions for the reactions were carefully studied. For acetaminophen, the reaction products with diazosulfanilic acid and diazo-*p*-nitroaniline show maximum absorbance at 480 and 425 nm, respectively. The mean percentage recoveries for authentic samples were 99.5 ± 1.1 and 100.6 ± 0.66, respectively (P = 0.05). For oxyphenbutazone, the obtained colors showed maxima at 385 nm with diazosulfanilic acid and 490 nm with diazo-*p*-nitroaniline reactions. The mean percentage recoveries for authentic samples were 99.8 ± 0.27 and 100.1 ± 0.57, respectively (P = 0.05). The proposed methods were successfully applied to the analysis of commercial preparations; results were statistically compared with those of other methods.

In view of the importance of acetaminophen as well as oxyphenbutazone as analgesic, antipyretic, and anti-inflammatory drugs, several colorimetric methods were reported for their determination. The majority of the published methods for acetaminophen depend on hydrolysis of the compound to *p*-aminophenol. The latter can then be estimated through its amino group either by diazotization and coupling (1, 2), condensation with certain aldehydes (3–6), nitration (7, 8), or oxidation (9). Other methods based on the phenolic nature of the drug depend on color reactions with Folin-Ciocalteu reagent (10) and nitrous acid (11–13). The reported color reactions for oxyphenbutazone depend either on hydrolysis followed by condensation with some aldehydes (14) or on direct reaction with 4-aminoantipyrine (15) or nitrous acid (16). Svatek and Hardkova (17) reported a direct method using reaction of oxyphenbutazone with diazotized sulfanilic acid, but the reported procedure was not reproducible in our laboratory.

The present communication describes direct color reactions in which the phenolic drugs are coupled with diazosulfanilic acid or diazo-*p*-nitroaniline. The procedure is simple, accurate, applicable to dosage forms, and suitable for routine analysis.

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Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Unicam SP1800 supplied with 1 cm glass cells.

(b) *Reagents.*—Methanol, chemically pure (Prolabo); 1N NaOH; 0.5% sodium nitrite in water; 0.2% sulfanilic acid in 1N phosphoric acid; 0.2% *p*-nitroaniline in 1N phosphoric acid.

All chemicals including acetaminophen and oxyphenbutazone are pure and pass BP (1973) requirements.

Sample Preparation

Transfer aliquot of crushed tablets, melted suppositories, syrups, or drops corresponding to ca 100 mg phenolic compound to 100 mL volumetric flask. Add 50 mL water, shake thoroughly (warm slightly for suppositories), and dilute to volume with water. Filter, if necessary, transfer 10 mL to 100 mL volumetric flask, and dilute to volume with water.

Procedure

Transfer 1 mL amine reagent (sulfanilic acid or *p*-nitroaniline) to 25 mL volumetric flask and add 0.5 mL sodium nitrite reagent. Mix thoroughly, and let stand at room temperature 10 min. Add 1 mL methanol and mix well to eliminate excess nitrous acid. Add 3 mL aqueous solution of phenolic compounds (equivalent to ca 0.3 mg), followed by 3 mL 1N NaOH. Concomitantly, prepare blank in which phenol is replaced by 3 mL distilled water. Dilute both solutions to volume with water and let stand at room temperature additional 20 min. Measure absorbance at maximum wavelength indicated below. Calculate concentration of sample from calibration graph similarly prepared by using known amounts of phenol tested.

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Phenol	Diazosulfanilic acid λ max.	a	Diazo-p-nitroaniline λ max.	a
Acetaminophen	480 nm	281	425 nm	299
Oxyphenbutazone	385 nm	287	490 nm	402

Results and Discussion

Acetaminophen couples with diazosulfanilic acid and diazo-*p*-nitroaniline to give red and yellow azo dye derivatives, respectively (Figure 1). Similarly, oxyphenbutazone couples with these reagents to give yellow and red azo dye derivatives, respectively (Figure 2). Because the para position is occupied in both compounds, coupling would occur in the ortho positions (18).

To study the stoichiometry of these reactions, we applied the method of continuous variation (19) in which the total molar concentration is kept constant and the mole fraction of one of the components is plotted on the abscissa scale. The ordinate scale represents the difference in absorbance reading (ΔA), i.e., the difference between the measured absorbance and the summed absorbances of the independent components. The resulting curve will show a maximum or minimum at the mole fraction corresponding to that in the reaction product. As evident from

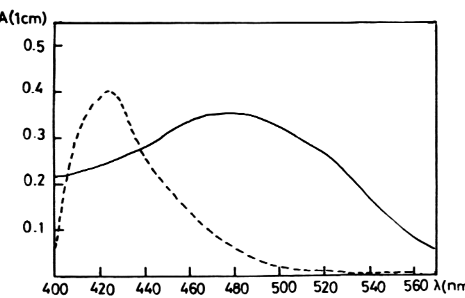

Figure 1. Absorption spectra of acetaminophen (12 µg/mL) coupling reaction with diazosulfanilic acid (—) and diazo-*p*-nitroaniline (- - -).

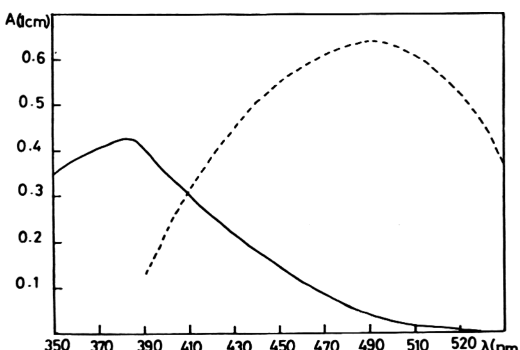

Figure 2. Absorption spectra of oxyphenbutazone (16 µg/mL) coupling reaction with diazosulfanilic acid (—) and diazo-*p*-nitroaniline (- - -).

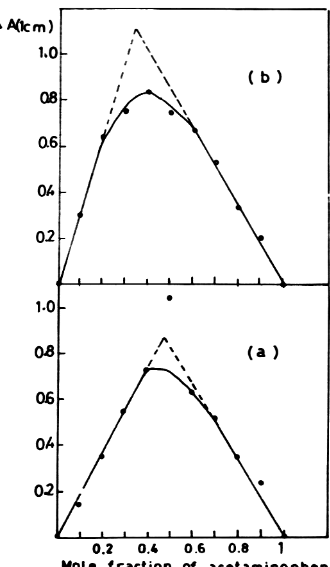

Figure 3. Continuous variation plots of acetaminophen with diazosulfanilic acid (a) and diazo-*p*-nitroaniline (b).

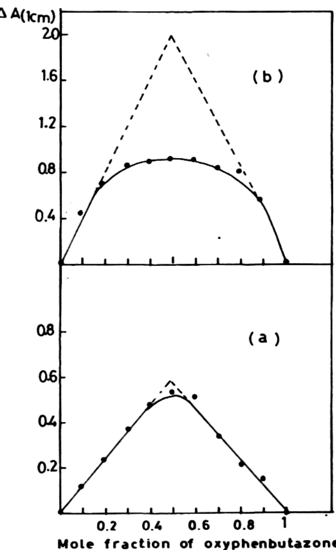

Figure 4. Continuous variation plots of oxyphenbutazone with diazosulfanilic acid (a) and diazo-*p*-nitroaniline (b).

Table 1. Comparison between proposed reactions for determining acetaminophen and oxyphenbutazone

Statistic	Diazosulfanilic acid		Diazo-*p*-nitroaniline
	Acetaminophen		
Number	9		8
Mean rec, % ($P = 0.05$)	99.5 ± 1.10		100.6 ± 0.66
Beer's law range (μg/mL)	2–20		4–18
CV, %	3.11		0.98
Student's *t*		1.55 (1.75)[a]	
F test		3.17 (3.6)[a]	
	Oxyphenbutazone		
Number	6		8
Mean rec., % ($P = 0.05$)	99.8 ± 0.27		100.1 ± 0.57
Beer's law range (μg/mL)	2–14		2–18
CV, %	0.11		0.73
Student's *t*		0.81 (1.782)[a]	
F test		6.64 (4)[a]	

[a] Theoretical values at $P = 0.05$.

Figure 3, the molar ratio (acetaminophen:diazoamine) is 1:1 with sulfanilic acid and 1:2 with *p*-nitroaniline: Each molecule of acetaminophen couples with one molecule of diazosulfanilic acid or with 2 molecules of diazo-*p*-nitroaniline. This may be due to the more electrophilic character of diazo-*p*-nitroaniline relative to diazosulfanilic acid (20).

The molar ratio oxyphenbutazone:diazoamine is 1:1 in both cases (Figure 4): One molecule of oxyphenbutazone couples with only one molecule of either diazosulfanilic acid or diazo-*p*-nitroaniline.

The effect of different parameters was studied and the optimum conditions were included in the given procedure. Phosphoric acid is preferred to hydrochloric acid (17), widely used in such reactions. When the medium is made alkaline with sodium hydroxide, phosphoric acid acts as a buffer due to the formation of sodium

Table 2. Application of proposed color reactions to determination of acetaminophen and oxyphenbutazone in pharmaceutical preparations

Preparation	Label, mg/dose unit	Found, % of label		
		Lit.[a]	Sulfan.	Nitroan.
		Acetaminophen		
Paracetamol tab, Misr Co.	500	101.3	97.1	100.0
Noflu tab,[b] Kahira Co.	400	98.6	97.1	98.7
Vegaskin tab,[c] Alex. Co.	200	109.0	101.5	105.3
Pyral drops, Kahira Co.	1500/5 mL	105.6	98.5	98.7
Paracetamol drops, Misr Co.	2400	102.8	97.1	98.7
Grippo supp., Nile Co.	200	100.0	95.6	96.0
Codacetine tab,[d] Kahira Co.	250	110.6	214.7	213.3
		Oxyphenbutazone		
Tandril tab, Ciba Co.	100	98.6	104.4	103.2
Oxyzone tab, Nile Co.	100	98.8	100.0	102.4
Rhumazin tab, Alex. Co.	100	98.5	98.5	100.8
Rhumaxin supp., Alex. Co.	250	96.5	97.1	98.4

[a] Literature method: nitrous acid for acetaminophen; 4-aminoantipyrine for oxyphenbutazone.
[b] Each tablet contains acetaminophen 400 mg, chlorpheniramine maleate 3 mg, and phenyl propanolamine hydrochloride 24 mg.
[c] Each tablet contains aspirin 300 mg, acetaminophen 200 mg, and codeine phosphate 10 mg.
[d] Each tablet contains salicylamide 300 mg, acetaminophen 250 mg, and codeine phosphate 10 mg.

phosphate and enhances reproducible results of the pH-sensitive coupling reactions. The developed colors are stable for several hours.

As evident from absorptivity values (*a*), reactions are of equal sensitivity with respect to acetaminophen determination. Results in Table 1 show that both reactions have comparable accuracy and precision according to calculated *t* values and *F* tests. On the other hand, the *p*-nitroaniline reaction is about 1.5 times more sensitive than the sulfanilic acid for oxyphenbutazone but less precise.

The proposed method was applied to the analysis of commercial preparations (Table 2). Statistical comparison of the results of the proposed method with the nitrous acid method (13) for the determination of acetaminophen, using the paired test (21), showed a nonsignificant difference. The exceptionally high results for Codacetine tablets are due to interference from salicylamide content. The difference between the results for oxyphenbutazone by the proposed method and the 4-aminoantipyrine method (15) is also nonsignificant. However, the present method is more sensitive than those previously reported.

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Chloridometer Determination of Alien Halide Content of Iodine

WILLIAM FERREN and LEONARD MALTESE[1]
Wagner College, Department of Chemistry, New York, NY 10301

A coulometric method is described for measuring alien halide content (usually bromide and chloride) of iodine crystals at levels less than 50 ppm. Our method replaces the vintage visual turbidimetric (Nessler tubes) measurement of the USP and ACS methods with an accurate, precise electronic end point of a chloridometer. The limit of detection is about 1 ng chloride, with an uncertainty of about 3%. Our procedure avoids the inherent errors associated with visual comparison between sample, blank, and control systems with widely differing indifferent ion populations.

The determination of alien halide content (usually as chloride or bromide) in iodine crystals is a matter of economic significance and a challenge to the analytical chemist. Methods in current use suffer from numerous problems, and resultant controversies and disputes have often been based on individual judgments regarding a vintage turbidimetric method end point. The words of Wells (1), "Apparently turbidity measurements have not proven satisfactory," written in 1927 apply to the methods of the USP and ACS (2, 3) which are currently most widely used in the determination of bromide or chloride content of iodine crystals. In contrast, our proposed method uses the electronic end point of the Cotlove chloridometer as a coulometric method. The accuracy and precision of this end point is well established by the universal use of the Cotlove chloridometer in applications ranging from the automatic, rapid, accurate, and sensitive determination of chloride described in Cotlove's original paper (4) to the use of the chloridometer in applications ranging from determination of potassium to determination of environmental organic halides (as opposed to ionic halides) described by Ferren et al. (5–8).

Experimental

Samples analyzed were iodine crystals. Weigh 1 g (±0.1 mg) sample and add to solution containing 600 mg hydrazine sulfate in 100 mL hot (80–95°C) water in 300 mL Erlenmeyer flask. Heat on steam bath until complete dissolution. Prepare blank by identical procedure except omit iodine crystals. Neutralize both sample and blank to same approximate pH by dropwise addition of 10% NaOH, using litmus paper as external indicator of color change from red to blue. Subsequently, add 1 mL concentrated phosphoric acid and 3 mL 30% hydrogen peroxide to both systems. Remove resultant iodine in sample system by vigorous boiling; then cool. Add another 1 mL 30% hydrogen peroxide to both flasks and if coloration takes place in the sample flask the residual iodine is boiled off completely. After oxidation and boiling processes have removed all iodine from sample system, dilute both systems to 150 mL with water. Remove all hydrogen peroxide from both systems by gently boiling down to 100 mL. Next, place 1 mL each of sample and blank in Buchler chloridometer vials containing 4 mL standard chloridometer titration solution. (This standard chloridometer solution is prepared by diluting 6.4 mL concentrated nitric acid and 100 mL glacial acetic acid to 1 L with distilled water.) Place 100 μL 0.01M NaCl solution in third vial as a useful and convenient reference standard. Add to each vial either gelatin or polyvinyl alcohol as electrode conditioner, as described in Buchler chloridometer instruction manual (9). Determine alien halide content of iodine crystal with Buchler digital chloridometer (Model 4-2500) by applying following formulas (high setting for high concentration levels and low setting for low concentrations):

At high setting:

Alien halide as chloride, ppm = [net readout (meq./L) $\times$ 35.5 $\times$ 10^{-1}]/g iodine crystals sample

Table 1. Coulometric determination of alien halide content of various commercial samples of iodine crystals (average of 3 determinations)

Sample	ng chloride/ g iodine[a]	max. rel. error, %[b]
A	5.7	0.77
B	4.5	1.03
C	11.9	0.97
D	23.8	0.66
E	7.7	0.96

[a] Average of 3 determinations.
[b] Based on maximum deviation from average.

[1] Stillwell and Gladding Testing Laboratories, New York, NY 10006.

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Table 2. Comparison of 3 methods for determining alien halide (as chloride)

Sample	ng chloride/g iodine			Rel. error, % (Based on proposed method)	
	Ferren-Maltese	USP	ACS	USP	ACS
F	6.6	5.2	7.2	21.2	9.0
G	29.9	22.7	35.7	24.0	19.4
H	25.2	19.5	31.2	22.6	23.4

At low setting:

Alien halide as chloride, ppm = [net readout (meq./L) × 35.5 × 10^{-2}]/g iodine crystals sample (see Figure 1).

Results and Discussion

A plot of read-out values as ng chloride (or ppb, based on a 1 g sample) vs added chloride, using an iodine sample at an initial level of 3.6 ppb as chloride, indicates a limit of detection of about 1 ng chloride, or an uncertainty of about 3%. Alien halide content (as chloride) of 5 commercial samples of iodine crystals was determined by triplicate analyses (Table 1). Comparison of results between our method and the USP and the ACS methods (2, 3) is shown in Table 2. The USP method depends on trituration to extract occluded alien halides (such as bromide and chloride); the ACS method uses hydrazine sulfate and heat. In our experience, both procedures work if proper care is given to technique. However, subsequent measurement steps specify visual turbidimetric techniques (Nessler tubes) in which the USP is superior because the sample and blank systems have much simpler and similar indifferent ion populations. The ACS method requires excess NaOH in the blank and excess nitric acid which results in a blank less than optimum for a visual turbidimetric measurement. In essence our method has combined the superior ACS extraction procedure with a coulometric measurement procedure.

The method reported here may prove useful in disputes between 2 existing commonly used methods, i.e., the USP method and the ACS method. It is hoped that other laboratories will consider the virtue of replacing a turbidimetric visual end point of dubious reproducibility with the coulometric technique which employs the Cotlove chloridometer, an instrument employed for many decades in routine analyses for chloride in a wide variety of areas of application.

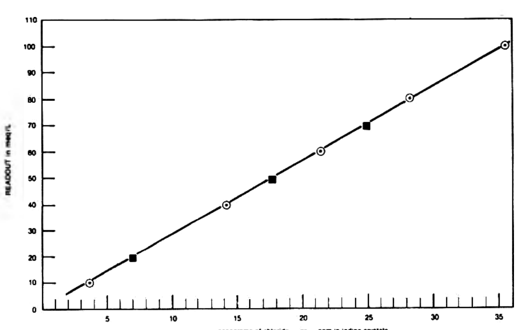

Figure 1. Calibration curve relating chloridometer readout to amount of alien halide (as chloride) in iodine crystals by Ferren-Maltese method: ■ aliquot volume of 0.01N NaCl; O iodine sample spiked with chloride.

Acknowledgment

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FOOD ADDITIVES

Improved 4-Aminoantipyrine Colorimetry for Detection of Residual Hydrogen Peroxide in Noodles, Fish Paste, Dried Fish, and Herring Roe

YOSHIO ITO, YASUHIDE TONOGAI, HIDEYO SUZUKI, SHUNJIRO OGAWA, TSUYOSHI YOKOYAMA,[1] TAKASHI HASHIZUME,[1] HIDEYUKI SANTO,[1] KEI-ICHI TANAKA,[2] KIKUYO NISHIGAKI,[2] and MASAHIRO IWAIDA
National Institute of Hygienic Sciences, Osaka Branch, 1-1-43, Hoenzaka, Higashi-ku, Osaka 540, Japan

Improved 4-aminoantipyrine (4-AA) colorimetry was developed for the detection of minute quantities of residual hydrogen peroxide in several kinds of food. Hydrogen peroxide in the sample was stabilized with potassium bromate and extracted with cold methanol. The methanol extract was diluted with phosphate buffer, protein was eliminated with zinc sulfate, and the extract was reacted with phenol, 4-AA, and peroxidase to the stable quinoneimine dye. The weak color solution was purified with Florisil column chromatography, concentrated, and determined by colorimetry. Recoveries of hydrogen peroxide from samples fortified at 0.5, 2, and 10 ppm ranged from 70.7 ± 8.9 to 98.5 ± 1.2%. Color development was linear with amount of H_2O_2 from 0.5 to 20 μg, corresponding to 0.05-2 ppm in samples.

Hydrogen peroxide has been used in Japan for a long time as a food additive for its antiseptic and bleaching properties. Because of the reported carcinogenicity to mice, the government of Japan made a partial amendment of the "Notification Regarding the Standards and Specifications of Food, Food Additives, etc." (Ministry of Health and Welfare Notification No. 370, December 28, 1959) to provide notification that hydrogen peroxide must be either decomposed or removed from final products (1). Residues must not exceed 0.1 ppm under the new regulation in effect since October 1, 1980. Accordingly, it became necessary to develop a sensitive method for the detection of residual hydrogen peroxide in foods.

A variety of methods have been reported (2–5); these methods, however, measure tens of parts per million hydrogen peroxide in foods, the lowest detection limit being between 5 and 10 ppm. We reported a 4-aminoantipyrine (4-AA) method (4) in which hydrogen peroxide was reacted with phenol, 4-AA, and peroxidase to produce the stable quinoneimine dye. We undertook to improve the 4-AA method so that it may be used to detect sub-ppm levels of residual hydrogen peroxide in foods.

[1] Wakayama Prefectural Institute of Public Health, Sunayama 3-chome, Wakayama, Japan.
[2] University of Kobe Gakuin, Faculty of Nutrition, Ikawadani, Tarumi-ku, Kobe, Japan.

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METHOD

Apparatus

(**a**) *Rotary evaporator.*—Model N-1 (Tokyo Rikakikai Co. Ltd, Tokyo, Japan), with ₮ 15/25 rotatory joint and Pyrex condenser.

(**b**) *Spectrophotometer.*—Bausch & Lomb Spectronic 20 (Shimadzu Seisakusho Ltd, Kyoto, Japan).

(**c**) *Chromatographic column.*—Glass, 1.5 cm id × 30 cm long, packed with 60–100 mesh Florisil (Katayama Kagaku Kogyo Ltd, Osaka, Japan) to height of 8 cm and washed with water until eluate is clear.

(**d**) *Buchner funnel.*—Set filter paper (No. 5C, Toyo Roshi Kaisha Ltd, Tokyo, Japan) on 5 cm id Buchner funnel, pour slurry of 5 g Celite 545 to make 5 mm layer, and wash with water.

Reagents

(**a**) *Methanol.*—Add ca 20 g $KBrO_3$ to 2 L methanol, shake 1 min, and keep in a refrigerator.

(**b**) *Zinc sulfate solution.*—Dissolve 10 g $ZnSO_4 \cdot 7H_2O$ in 100 mL water.

(**c**) *Phenol solution.*—Dissolve 2.0 g phenol in 100 mL water. Prepare fresh before use.

(**d**) *4-AA solution.*—Dissolve 400 mg 4-AA in 100 mL water.

(**e**) *Peroxidase solution.*—Dissolve 10 mg peroxidase (P-L Biochemicals, Inc., Milwaukee, WI, ca 200 units/mg) in 100 mL water. Prepare fresh before use.

(**f**) *Phosphate buffer.*—0.5M, pH 7.3. Dissolve 68 g KH_2PO_4 in 1 L water; adjust pH to 7.3 with

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solution prepared by dissolving 179 g $Na_2HPO_4 \cdot 12H_2O$ in 1 L water.

(g) *Celite 545.*—Add 200 g Celite 545 (Nakarai Chemicals Ltd, Kyoto, Japan) to 200 mL HCl and 500 mL water, heat 1 h at 100°C, filter with suction, wash with water until pH of eluate reaches ca 6–7, dry >10 h at 110°C, and crush for use.

(h) *Standard hydrogen peroxide solutions.*—(*1*) *Stock solution.*—1 mg H_2O_2/mL. Dilute 1.0 mL 30% H_2O_2 (special grade supplied from Mitsubishi Gas Kagaku Ltd, Tokyo, Japan) in methanol to make 100 mL. Store solution in refrigerator and use within 1 month. (*2*) *Working solution.*—10 μg H_2O_2/mL. Mix 10 mL stock solution and 20 mL methanol. Transfer 1.0 mL of above solution to 100 mL volumetric flask, dilute to volume with methanol, and mix. Prepare fresh before use.

(i) *Samples.*—Cut noodle (Namamen), fish paste (Kamaboko), dried fish (white bait, young sardines called Shirasu), and herring roe (Kazunoko) into pieces.

Procedure

Extraction of hydrogen peroxide.—Weigh 20 g sample and 10 g potassium bromate into 200 mL blender cup, blend well with spoon, add 50 mL cold methanol, cool in ice-water bath, and blend 3 min. Centrifuge at 3000 rpm for 10 min, and collect methanol layer (supernate). Shake residue with 30 mL cold methanol for 3 min, and centrifuge. Combine methanol layers and dilute to 100 mL with cold methanol.

Removal of proteins.—Pipet 50 mL methanol extract into 300 mL conical flask, add 100 mL water, 22 mL phosphate buffer, and 15 mL zinc sulfate solution, mix, and let stand, swirling occasionally. After 10 min, add 5 g Celite 545, mix, and let stand 5 min. Then add 25 mL phosphate buffer, mix, immediately filter with suction through Buchner funnel, and wash with 30 mL water. Combine filtrates and dilute to 250 mL with water.

Color development.—Place 125 mL of above solution into 300 mL conical flask, add 3 mL phenol solution, 1 mL 4-AA solution, and 1 mL peroxidase solution, and react at 37°C for 15 min.

Removal of excess color reagents and coexisting substances.—Pour weakly colored solution onto column (1.5 × 8 cm) and let pass through at flow rate of 15 mL/min. Rinse column with 50 mL water–methanol (8 + 2); then elute quinoneimine dye (colored compound) from column with 50 mL acetone at flow rate of 5 mL/min. Concentrate to ca 3 mL at 80°C in a rotary evaporator under vacuum, dilute to 5.0 mL with water, and filter with Millipore® filter (pore size, 0.45 μm).

Color determination.—Measure absorbance of solution at 505 nm. Calculate hydrogen peroxide content in sample, expressed as ppm, with aid of calibration curve.

Table 1. Comparison of stabilizing reagents of hydrogen peroxide during methanol extraction

Reagent[a]	H_2O_2 added, ppm: 0	H_2O_2 added, ppm: 2	Recovery, %
Without reagent	ND[b]	0.21	10.6
EDTA	ND	ND	0.0
$KBrO_3$	ND	1.55	77.5
$NaClO_4$	ND	1.23	59.6
KI	ND	0.10	5.0
$NaNO_2$	ND	0.25	12.3
$K_2Cr_2O_7$	2.24	2.74	25.0
$FeCl_3$	2.30	4.38	104.2
$KMnO_4$	4.47	5.87	70.0

[a] Two hundred mg of each reagent was used per 20 g sample (dried fish).
[b] ND = <0.05 ppm.

Preparation of Standard Curve

Pipet 0, 0.2, 0.5, 1.0, 1.5, and 2.0 mL standard solution into 300 mL conical flask and dilute to 50 mL with cold methanol. Carry out analysis, starting with *Removal of proteins.*

Results and Discussion

It was necessary to use purification and concentration procedures to determine low levels of hydrogen peroxide in foods. However, residual hydrogen peroxide is unstable and will decompose during these procedures. Therefore, it was necessary to prepare a stable color derivative after extraction, which could then be purified, concentrated, and determined by colorimetry.

The quinoneimine dye produced from hydrogen peroxide, phenol, 4-AA, and peroxidase is one of the most stable compounds among color derivatives of hydrogen peroxide (3, 5). The original 4-AA method consisted of 3 steps: Hydrogen peroxide was extracted with 50 mL methanol from 10 g food; 0.5 mL extract was reacted 15 min with 5 mL color reagents containing phenol, 4-AA, and peroxidase at 37°C; then absorbance of the solution was measured at 505 nm. The detection limit of hydrogen peroxide by this method, however, was 10 ppm.

In our attempts to improve this method, we used dried fish, which is one of the most difficult

Table 2. Effect of filter aids on filtration with suction [a]

Filter aid	Filtration time, min	A, 505 nm
Celite 545	3	0.003
Avicel	4	0.008
Diatomaceous earth	5	0.010
Cellulose powder	9	0.021
Activated clay	14	0.090
Talc	19	0.005
Permutit	20	0.048
Without filter aid	30	0.300

[a] Fifty mL methanol extract of dried fish was diluted with 100 mL water, 22 mL phosphate buffer and 15 mL zinc sulfate solution was added, and mixed. After 10 min standing, 5 g filter aid was added. After 5 min, 25 mL phosphate buffer was added, and filtered with suction through a Buchner funnel. Filtration speed and clearance of filtrate expressed by *A* at 505 nm are indicated.

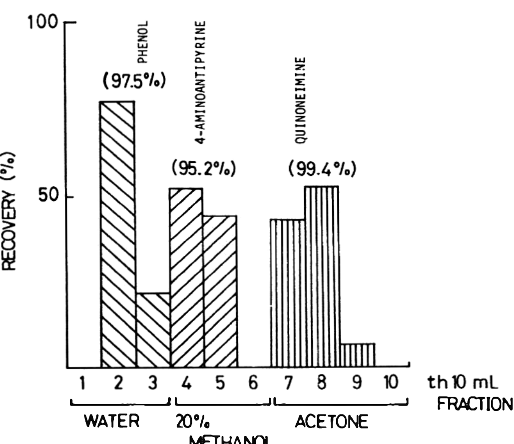

Figure 2. Recovery of dye components vs elution volume required using Florisil column.
Ten milliliter of mixture containing 60 mg phenol, 4 mg 4-aminoantipyrine, and 5 mg quinoneimine dye was used. Phenol in each fraction was determined by FID-GC method, and 4-aminoantipyrine and quinoneimine dye were determined by visible absorption method.

samples for determination of residual hydrogen peroxide because of the large amounts of lipids, proteins, and salts. The sample was fortified with 2 ppm hydrogen peroxide and homogenized with cold methanol for extraction under ice-cold conditions. About 90% of the added hydrogen peroxide decomposed in this procedure, probably due to reducing components.

Several reagents were added into the sample, mixed well with a spoon, and then homogenized with cold methanol. Table 1 shows that potassium bromate most effectively protects hydrogen peroxide during methanol extraction, with no interference in the color reaction. The amount of potassium bromate and recoveries of hydrogen peroxide were studied, and 10 g potassium bromate was adopted as the stabilizing reagent.

The small quantity of protein co-extracted into the methanol extract interfered with the color determination. The methanol extract was diluted with water, and phosphate buffer (pH 7.3) was added to stabilize hydrogen peroxide and to adjust pH for protein precipitation (pH 5.5–6.2) by addition of zinc sulfate (pH 1.0); the precipitate was filtered with suction. The filtering operation was quite slow, so filtering aid was used to accelerate the filtering rate and to obtain a clear filtrate (Table 2).

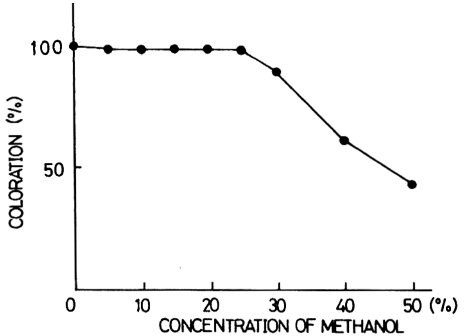

Figure 1. Effect of concentration of methanol on color reaction.

The presence of excess methanol in the filtrate interferes with an accurate determination of hydrogen peroxide by 4-AA colorimetry, because it interrupts enzymic reaction. Figure 1 shows that the color development is not interrupted below methanol concentrations of 25%. Accordingly, 50 mL methanol extract was diluted with 200 mL water. Half of the diluted solution (125 mL) was reacted with color reagents.

We attempted to concentrate the weakly colored solution to 5 mL at 80°C under vacuum. However, the reagent blank changed to a weak pink color from excess phenol reagent. Florisil column chromatography was good for removing phenol, because the quinoneimine dye is strongly adsorbed on Florisil. When 60 mg phenol, 4 mg 4-AA, and 5 mg quinoneimine dye were eluted through Florisil, 97.5% phenol was eluted with 30 mL water, 95.2% 4-AA was eluted with 30 mL 20% methanol, and 99.4% quinoneimine dye was eluted with 40 mL acetone (Figure 2).

Table 3. Recoveries of added hydrogen peroxide from various foods[a]

Food	H_2O_2 added, ppm	H_2O_2 found, ppm	Recovery, %
Noodle (Namamen)	0	ND	—
	0.5	0.41 ± 0.02	82.0 ± 4.6
	2.0	1.85 ± 0.07	92.5 ± 3.5
	10.0	9.85 ± 0.12	98.5 ± 1.2
Fish paste (Kamaboko)	0	0.12 ± 0.05	—
	0.5	0.51 ± 0.03	78.5 ± 5.3
	2.0	1.86 ± 0.08	86.9 ± 4.1
	10.0	9.91 ± 0.20	97.9 ± 2.0
Boiled, dried larval fish (Shirasu-boshi)	0	0.24 ± 0.04	—
	0.5	0.64 ± 0.04	80.6 ± 7.7
	2.0	2.03 ± 0.08	89.4 ± 4.2
	10.0	9.82 ± 0.32	95.8 ± 3.2
Herring roe (Kazunoko)	0	0.52 ± 0.09	—
	0.5	0.88 ± 0.04	70.7 ± 8.9
	2.0	2.23 ± 0.08	85.7 ± 4.0
	10.0	10.16 ± 0.29	96.4 ± 2.9

[a] Data show the mean values ± SD of 5 trials.

Table 4. Hydrogen peroxide content in various commercial foods

Sample	No.	H_2O_2, ppm Trial 1	2	3	Mean ± SD
Noodle	1	ND[a]	ND	ND	ND
	2	ND	ND	ND	ND
	3	ND	ND	ND	ND
	4	ND	ND	ND	ND
	5	0.05	0.06	0.06	0.06 ± 0.01
	6	0.07	0.09	0.11	0.09 ± 0.02
	7	0.10	0.11	0.13	0.11 ± 0.02
	8	0.62	0.65	0.66	0.64 ± 0.02
	9	1.25	1.25	1.26	1.25 ± 0.00
	10	1.50	1.51	1.51	1.51 ± 0.01
Fish paste	1	ND	ND	ND	ND
	2	0.08	0.09	0.09	0.09 ± 0.01
	3	0.15	0.18	0.21	0.18 ± 0.03
	4	0.19	0.21	0.23	0.21 ± 0.02
	5	0.27	0.28	0.29	0.28 ± 0.01
	6	0.29	0.29	0.29	0.29 ± 0.00
	7	0.57	0.59	0.60	0.59 ± 0.02
	8	0.71	0.72	0.72	0.72 ± 0.01
	9	1.05	1.06	1.03	1.32 ± 0.02
	10	1.22	1.22	1.25	1.23 ± 0.02
Boiled, dried larval fish	1	ND	ND	ND	ND
	2	0.15	0.16	0.16	0.16 ± 0.01
	3	0.16	0.19	0.21	0.19 ± 0.03
	4	0.20	0.20	0.22	0.21 ± 0.01
	5	0.16	0.20	0.21	0.19 ± 0.03
	6	0.24	0.29	0.30	0.28 ± 0.03
	7	0.25	0.31	0.34	0.30 ± 0.05
	8	0.42	0.45	0.45	0.44 ± 0.02
	9	0.76	0.79	0.79	0.78 ± 0.02
	10	1.20	1.21	1.22	1.21 ± 0.01

[a] ND = 0.05 ppm.

A straight line was obtained for color development and amount of hydrogen peroxide from 0.5 to 20 μg. This corresponds to 0.05–2 ppm H_2O_2 in samples.

Hydrogen peroxide was recovered from noodles, fish paste, dried fish, and herring roe at different fortification levels; results are shown in Table 3. Recovery was satisfactory at 10 ppm, and as high as 85% even at a low level of 2 ppm.

This established method is successfully applied to the daily inspection of commercial foods in our institute (Table 4).

Acknowledgment

This investigation was financially supported by the Scientific Research Fund of the Ministry of Health and Welfare of Japan, which is gratefully acknowledged.

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High Pressure Liquid Chromatographic Determination of Ethoxyquin in Paprika and Chili Powder

GRACIA A. PERFETTI, CHARLES R. WARNER, and THOMAS FAZIO
Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

A method is described for the determination of ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) in paprika and chili powder. Ethoxyquin is extracted from the spice with hexane and partitioned into 0.3N HCl. After adjusting the solution to pH 13-14, ethoxyquin is extracted into hexane, and the hexane layer is evaporated to dryness. An acetonitrile solution of the residue is then analyzed by reverse phase high pressure liquid chromatography with detection at 254 nm. The mobile phase is water-acetonitrile with ammonium acetate buffer. Recoveries from samples fortified at 50, 100, and 200 ppm averaged 92% with a coefficient of variation of 2.3%. The method was applied to a number of commercial samples of paprika and chili powder. Ethoxyquin was found in paprika samples at levels up to 63 ppm and in chili powder samples at levels up to 20 ppm.

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) is used as an antioxidant to retard decomposition of some pigments and fat-soluble vitamins in certain foods and animal feeds. In the United States, ethoxyquin is permitted at levels up to 150 ppm in animal feeds and dehydrated forage crops, and up to 100 ppm in paprika and chili powder. Tolerances are also specified for ethoxyquin residues in milk, eggs, and animal tissues.

No methods could be found in the literature for the determination of ethoxyquin in spices, but several were found for determining ethoxyquin in feeds and animal tissues. Most of these methods are based on either fluorescence or ultraviolet (UV) spectroscopy. The official AOAC methods for determining ethoxyquin in animal feeds (1) and in chicken tissues and eggs (2) are both based on the fluorescent nature of ethoxyquin. Fluorescence methods have also been employed by Bickoff et al. (3) and Witt et al. (4). Choy et al. (5) used UV spectrometry to determine ethoxyquin. All of these methods lack speed and specificity. Dahle and Skaare (6) determined ethoxyquin in fish and fish meal by gas-liquid chromatography. While the method possessed a degree of specificity, recoveries were unacceptably low, probably due at least in part to thermal decomposition of ethoxyquin.

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High pressure liquid chromatography (HPLC) is a logical alternative to the methods used previously for the determination of ethoxyquin. This highly selective technique may be carried out at ambient temperature, so that the thermal instability of ethoxyquin is not a problem. In addition, ethoxyquin is easily detected by conventional LC detectors. This paper describes an HPLC method for the determination of ethoxyquin in paprika and chili powder. The antioxidant is extracted from the spice with hexane, partitioned into acidic solution, and then partitioned back into hexane following pH adjustment. The hexane solution is evaporated to dryness, and the residue is dissolved in acetonitrile. The resulting solution is analyzed by reverse phase HPLC with detection at 254 nm.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Spectra-Physics Model 3500B operated at ambient temperature and flow rate of 1.2 mL/min (Spectra-Physics, Piscataway, NJ 08854).

(b) *Injector.*—Valco 7000 psig sample injection valve fitted with 10 μL sample loop (Valco Instruments Co., Inc., Houston, TX 77055).

(c) *Column.*—4.6 mm id × 250 mm stainless steel column packed with Zorbax ODS (E.I. du Pont de Nemours & Co., Wilmington, DE 19898).

(d) *Detector.*—Schoeffel Model 770 variable wavelength UV detector (Kratos, Inc., Westwood, NJ 07675). Sensitivity range 0.01-2 absorbance units full scale (AUFS); 8 μL flow cell. Wavelength set at 254 nm, time constant set at zero. Chromatograms were recorded on 10 mV strip chart recorder.

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

(f) *Sample filtration apparatus.*—Swinny type, used with 1 μm Fluoropore filters (Millipore Corp., Bedford, MA 01730).

(g) *Centrifuge.*—Sorvall RC-3 (du Pont Co.) or centrifuge capable of relative centrifugal force of 65.

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Reagents

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

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

(c) *Ethoxyquin.*—Technical grade, 90% (Pfaltz & Bauer, Inc., Stamford, CT 06902).

(d) *Ammonium acetate solution.*—0.01N. Dissolve 0.77 g in 1 L water (pH $\simeq$ 7).

(e) *Mobile phase.*—30% (by volume) ammonium acetate solution in acetonitrile.

(f) *Standard solutions.*—*Stock solution I.*—2.5 mg/mL. Accurately weigh 0.1 g ethoxyquin and transfer to 50 mL volumetric flask; dissolve and dilute to volume with acetonitrile. *Stock solution II.*—250 μg/mL. Pipet 5 mL stock solution I into 50 mL volumetric flask and dilute to volume with acetonitrile. *Working solutions.*—5, 15, 25, and 35 μg/mL. Pipet 1, 3, 5, and 7 mL stock solution II into separate 50 mL volumetric flasks and dilute to volume with acetonitrile.

Determination

Extraction.—Accurately weigh 2 g spice into 15 mL capped centrifuge tube. Extract with three 10 mL portions of hexane. Centrifuge at 500 rpm 5 min after each extraction to settle particulates. Decant and combine extracts in 125 mL separatory funnel, add 15 mL 0.3N HCl, and shake *gently* 1 min. Let layers separate completely; then transfer aqueous (lower) layer to 60 mL separatory funnel. Extract hexane solution with another 15 mL 0.3N HCl, and transfer aqueous layer to 60 mL funnel. Add 2 mL 4.8N NaOH to 60 mL funnel and *immediately* extract with 10 mL hexane. Draw off aqueous (lower) layer and discard. Drain hexane layer into 100 mL round-bottom flask. Rinse 60 mL separatory funnel with 5 mL hexane and add to flask. Add 5 mL acetonitrile to flask (to aid in removal of residual water) and evaporate to dryness on flash evaporator, using 35°C water bath. Dissolve residue in 10 mL acetonitrile. Filter through 1 μm Fluoropore filter.

Chromatography.—Set detector range to 0.1 AUFS. Inject 10 μL sample solution and 4 working solutions. If sample peak is larger than peak for most concentrated standard, accurately dilute sample solution. If sample peak is smaller than peak for least concentrated standard, prepare 2.5 μg/mL standard and inject 10 μL aliquot. If sample peak is smaller than this, prepare another sample as above, but dissolve residue in smaller amount acetonitrile (minimum 2 mL).

Calculation

Determine peak heights for sample and standards. Plot peak heights vs concentration for standards and determine slope (S) and intercept (I) of line by performing linear least squares analysis. Calculate concentration of ethoxyquin in sample as follows:

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

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

Results and Discussion

Initial attempts to chromatograph ethoxyquin by reverse phase HPLC resulted in broad, asymmetric peaks with long and variable retention times. Such behavior has been observed previously for amines in general, and has been attributed to interactions between the amine functional group and non-bonded silanol groups on the packing material. These interactions may be eliminated by adding an acidic buffer containing either an alkyl ammonium compound (7) or an alkyl amine (8) to the mobile phase. We found ammonium acetate solution to be just as effective for this purpose, and simpler to prepare. Figure 1 shows chromatograms of ethoxyquin with and without ammonium acetate in the mobile phase.

Ethoxyquin absorbs UV light and fluoresces; thus it is easily detected by 2 conventional LC detectors. Since the tolerance level for ethoxyquin in paprika and chili powder is high (100 ppm), we chose to use the less sensitive UV detector. At 254 nm, with the conditions specified above, the noise level of the detector was about 2×10^{-4} AU. The least concentrated ethoxyquin working solution (2.5 μg/mL) gave a response of about 4×10^{-3} AU, or 20 times noise level. Although higher sensitivity can be obtained at the absorption maximum of 230 nm, we used 254 nm to enhance specificity. A spice sample containing 2.5 ppm ethoxyquin would give rise to a sample solution with a concentration of about 2.5 μg/mL (assuming a final volume of 2 mL). Therefore, in the absence of any chromatographic interferences, the method can be applied to spice samples containing as little as 2.5 ppm ethoxyquin.

Almost any organic solvent can be used to quantitatively remove ethoxyquin from paprika and chili powder. However, these extracts contain many UV-absorbing materials, making

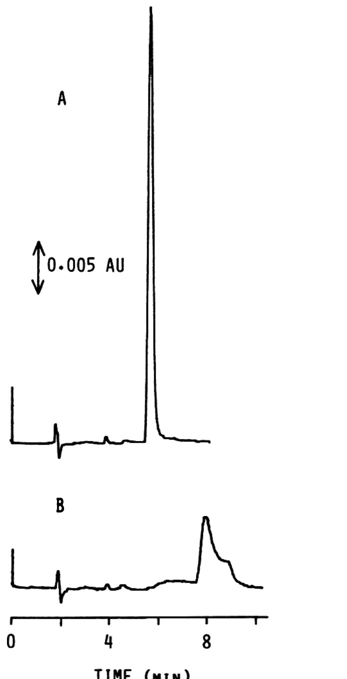

Figure 1. Chromatograms of 25 μg/mL ethoxyquin working solution. A, 30% 0.01N ammonium acetate-70% acetonitrile as mobile phase; B, 30% water-70% acetonitrile as mobile phase.

the HPLC separation difficult. Hexane was chosen as the extracting solvent for two reasons. First, it was found that ethoxyquin could easily be removed from hexane extracts of the spices by shaking with dilute hydrochloric acid. Ethoxyquin, which is weakly basic, partitions into the aqueous phase, whereas most of the impurities remain in the organic phase. Emulsions were sometimes encountered upon shaking the hexane extracts with hydrochloric acid. By shaking the phases together very gently, the formation of stable emulsions could be avoided. The second reason for choosing hexane was the high stability of ethoxyquin in this solvent. Ethoxyquin decomposes in many solvents, particularly when exposed to light. For example, solutions of ethoxyquin in methanol and methylene chloride deteriorate rapidly when stored in light. On the other hand, hexane solutions are stable for several days in lighted areas and for several weeks in the dark.

Although the hydrochloric acid solution of ethoxyquin was very clean, it could not be injected into the liquid chromatograph because of incompatibility with the HPLC mobile phase. Thus, another step was added to the method in which the hydrochloric acid solution was made basic, and ethoxyquin partitioned back into hexane. (Since ethoxyquin can decompose in basic solution, the hexane extraction should be performed immediately following addition of sodium hydroxide.) When the hexane was removed on a flash evaporator, traces of water remained in the flask. To avoid a volume error, the residue had to be evaporated completely to dryness. Addition of 5 mL acetonitrile to the hexane solution led to complete removal of solvent.

The sample was prepared for HPLC by dissolving the residue in acetonitrile. Not only is acetonitrile compatible with the HPLC mobile phase, but the stability of ethoxyquin in this solvent is almost as high as in hexane. To avoid errors due to decomposition, the following precautions were taken. Sample and standard solutions were stored in the dark when not in use. Sample solutions were analyzed the same day they were prepared, and standards were prepared fresh weekly.

Using the described method, recovery studies were carried out at 50, 100, and 200 ppm (one-half, one, and two times tolerance level) in paprika and chili powder. Sample chromatograms are shown in Figures 2 and 3. A sample was prepared by spiking 2 g spice in the centrifuge tube with 1 mL hexane stock solution and mixing with a vortex mixer. For each spice, 10 samples at each level were prepared and analyzed. Results are summarized in Table 1. The average recovery for all fortified samples was 92%, with a coefficient of variation (CV) of 2.3%.

The lack of purity of the technical grade eth-

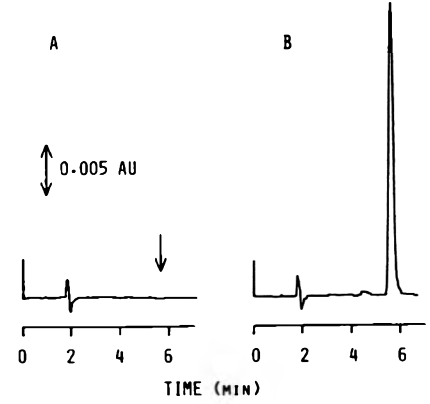

Figure 2. Chromatograms of A, paprika containing no ethoxyquin; B, paprika spiked with 100 ppm ethoxyquin.

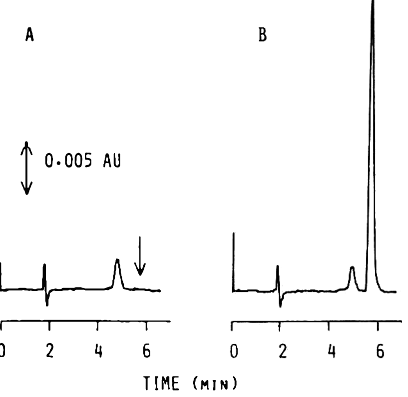

Figure 3. Chromatograms of A, chili powder containing no ethoxyquin; B, chili powder spiked with 100 ppm ethoxyquin.

Table 2. Ethoxyquin found (ppm) [a] in commercial paprika and chili powder

Brand	Lot 1	Lot 2
	Paprika	
A	16	26
B	6	6
C	30	63
D	46	48
E	0	— [b]
F	8	—
	Chili Powder	
A	3	20
B	5	15
C	0	4
D	5	15
E	14	—
F	15	—

[a] Average of duplicate determinations.
[b] Not available for analysis.

oxyquin used in the method had no effect on the accuracy of the recovery data, since the same material was used to prepare both the spiking solutions and the standard solutions. But in order to use this material as a standard in the analysis of commercial paprika and chili powder, we had to accurately determine its ethoxyquin content. We assayed the technical grade material by HPLC using the hydrochloride salt of ethoxyquin as a reference standard material. The salt was prepared in a manner analogous to that used by Craig (9). Briefly, 10 g technical grade ethoxyquin was vacuum distilled, and the fraction boiling at 111–115°C (P = 0.2 torr) was collected. The liquid was dissolved in 75 mL ethyl ether and shaken with 5 mL concentrated HCl. Crystals of ethoxyquin hydrochloride began to form almost immediately. The crystals were filtered and recrystallized from 3N HCl. Standard solutions of the salt for use in the assay were prepared using 10% (by volume) water in acetonitrile as the solvent. The average ethoxyquin content of the technical grade material from 8 determinations was 81.4% (CV = 0.6%).

The described method was applied to a number of commercial brands of paprika and chili powder. The results are presented in Table 2. In all, 10 samples of each spice were analyzed. Ethoxyquin was found in all but one of the paprika samples at levels up to 63 ppm, and in all but one of the chili powder samples at levels up to 20 ppm. In many cases, different lots of the same brand of spice had different ethoxyquin contents.

Table 1. Recovery (%) [a] of ethoxyquin from paprika and chili powder

Added, ppm	Paprika	Chili powder
50	92 (1.8)	91 (2.1)
100	92 (2.0)	90 (1.9)
200	95 (1.3)	92 (1.8)

[a] Each value is based on 10 determinations. CV in parentheses.

In summary, the described HPLC method can be used to determine ethoxyquin in paprika and chili powder. With minor modification it should also be applicable to the determination of ethoxyquin in animal feeds. By using the more sensitive fluorescence detection, the method could also be modified to determine ethoxyquin in eggs, milk, and animal tissues.

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Determination and Confirmation of *N*-Nitrosodimethylamine in Beer

DENIS ANDRZEJEWSKI, DONALD C. HAVERY, and THOMAS FAZIO
Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

A qualitative method has been developed for the confirmation of *N*-nitrosodimethylamine (NDMA) in beer by capillary gas chromatography-mass spectrometry (GC/MS) from full mass scans after quantitation, using a gas chromatograph interfaced to a thermal energy analyzer (GC/TEA). The GC/MS method has a lower limit of confirmation of 5 ppb. In a survey of 22 imported and 42 domestic beers, NDMA was found in 60 samples by GC/TEA at levels up to 7.7 ppb, and was confirmed by GC/MS in 5 samples.

Volatile *N*-nitrosamines, a class of potent carcinogens, have been found in a variety of food products (1, 2). In 1979, Spiegelhalder et al. (3), while screening foods obtained from the German market, found *N*-nitrosodimethylamine (NDMA) in 70% of the 158 samples of commercially available beers analyzed. These investigators, using a thermal energy analyzer (TEA), determined that the average level of NDMA in beers was 2.7 ppb. Confirmation of the identity of the *N*-nitrosamine was based on high resolution mass spectrometric data at 12 000 resolution for samples containing NDMA at levels greater than 3 ppb. The finding of NDMA in beer prompted the Food and Drug Administration to examine a variety of beers sold in the United States. We report here the results of a survey of domestic and imported beers for NDMA, in which a gas chromatograph interfaced to a TEA (GC/TEA) was used for screening and quantitation. The identity of NDMA was confirmed by gas chromatography—mass spectrometry (GC/MS).

Some detection methods commonly used for nitrosamine analysis may give rise to false-positive results. In 1968, McGlashan et al. (4) reported finding NDMA in African potable spirits by polarography and thin layer chromatography. Subsequent examination of the suspected nitrosamine by GC/MS showed that the compound was in fact furfural (5, 6). It is now generally accepted that mass spectral analysis is essential for unequivocal confirmation of the identity of nitrosamines in a sample.

Several recently published reports have described various mass spectral techniques used to confirm the identity of NDMA in beer. Goff and Fine (7) used high resolution GC/MS in the analysis of 18 brands of domestic and imported beers; peak matching of the NDMA molecular ion against a standard at 10 000 resolution was used as their confirmatory method. Sen et al. (8) examined a variety of Canadian and imported beers in which GC/TEA analysis indicated the presence of NDMA in 21 of the 22 beers tested. Their GC/MS method involved single ion monitoring of the NDMA molecular ion at 5000 resolution. The levels of nitrosamine reported ranged from 0.4 to 4.9 ppb. In 1980, Scanlan et al. (9) examined a variety of commercially available beers produced in the United States; NDMA was detected in 23 of the 25 beers tested and the mean level was 5.9 ppb. The confirmatory method used involved the repetitive trapping of NDMA from a beer extract during GC/TEA analysis followed by desorption of the trapped nitrosamine into a GC/MS system. Confirmation was then based on the full mass scan data at low resolution. In this report, we describe a method that provides for confirmation of the identity of NDMA in beer samples by low resolution capillary GC/MS from full mass scan data. A single, cleaned-up extract of each beer, which required no repetitive trapping before MS analysis, was used.

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METHOD

Apparatus

(a) *Boiling flasks.*—250 and 500 mL with 24/40 joint (K-601000, Kontes Glass Co., Vineland, NJ 08360).

(b) *Adapter.*—Connecting type with 24/40 joints and 10/30 top joint (Kontes, K-167000) and pennyhead stopper (Kontes, K-851000).

(c) *Condenser.*—Leibig, 200 mm with 24/40 joints (Kontes, K-447000).

(d) *Adapter.*—Bent 105° with 24/40 joint (Kontes, K-157000).

(e) *Evaporative concentrators.*—Kuderna-Danish, 250 and 500 mL, 24/40 column connection, 19/22 lower joint (Kontes, K-570001).

(f) *Concentrator tube.*—Size 425, 4 mL, 19/22 joint (Kontes, K-570050), with 19/22 pennyhead stopper (Kontes, K-850500).

(g) *Distilling column.*—Snyder, 3-section, size 121, 24/40 joint (Kontes, K-503000).

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(h) *Funnel.*—Buchner, 60 mL, with coarse porosity disk (Kontes, K-955000).

(i) *Chromatographic column.*—Chromoflex, size 241, 22 mm id × 300 mm (Kontes, K-420530).

(j) *Sample tube.*—Chromoflex, 10/18 joint (Kontes, K-422560), with stopper.

(k) *Gas chromatograph-thermal energy analyzer.*—Fisher/Victoreen (Fisher Scientific Co., Pittsburgh, PA 15219) series 4400 with Model 4006-14 auto programmer, Model FS01W6D strip chart recorder (0.5 in./min chart speed), Model 4002-12 oven, and Model 4020-1 temperature/indicator control, interfaced to Model 502L thermal energy analyzer (Thermo Electron Corp., Waltham, MA 02154). Operating conditions: 2.7 m × 4 mm id coiled glass column packed with 10% Carbowax 1540 + 5% potassium hydroxide on 100–120 mesh Chromosorb WHP; carrier gas (argon), 40 mL/min; temperatures, injection port 200°C, column isothermal 150°C, TEA furnace 450°C; pressure 1.2 torr; liquid nitrogen cold trap.

(l) *Gas chromatograph-mass spectrometer.*—Hewlett-Packard Model 5992A (Palo Alto, CA 94304) quadrupole mass spectrometer with HP5700A series gas chromatograph and HP18947A capillary column interface. Operating conditions: 40 m × 0.5 mm id Carbowax 20M capillary SCOT column; temperatures, injection port 200°C, oven 60 to 180°C at 12°/min following initial hold at 60°C for 8 min; carrier gas, helium, 3 mL/min. Hewlett-Packard magnetic tape software program (05992-10015, Peakfinder for Normalized Spectra) was used to calibrate and autotune the mass spectrometer in the electron impact mode at 70 eV. Full mass scans were acquired over the mass range m/z 25 to 150; scan rate 200 amu/s. Data were acquired and stored on the cartridge tape unit.

Reagents

(a) *Dichloromethane (DCM).*—Distilled-in-Glass (Burdick and Jackson Laboratories, Muskegon, MI 49442).

(b) *Celite 545.*—No. C-212, not acid washed (Fisher). Heat contents of each bottle overnight at 700°C before use.

(c) *n-Pentane.*—Redistill before use (J.T. Baker, Phillipsburg, NJ 08865).

(d) *N-Nitrosodimethylamine (NDMA).*—Serially dilute stock solution (1 mg/mL) to working standard (0.5 μg/mL) with DCM. Caution: NDMA is potent animal carcinogen and must be handled appropriately.

Each lot of reagent must be checked for interferences by performing blank analyses, substituting 25 g distilled water for sample.

Procedure

Weigh 25 g beer into 250 mL round-bottom flask containing 4 g barium hydroxide and boiling chips. Distill slowly (variable transformer 60%), collecting ca 24 mL distillate in 125 mL separatory funnel. Add 0.2 g anhydrous sodium carbonate. Extract distillate with four 10 mL portions of DCM, shaking each 1 min. Pass pooled extracts through 30 g anhydrous Na_2SO_4 (held in 60 mL fritted glass funnel and prewetted with DCM) into 250 mL Kuderna-Danish evaporative concentrator (KD) with 4 mL concentrator tube attached. Wash Na_2SO_4 with 15 mL DCM into KD. Add carborundum grains, attach 3-section Snyder column and carefully concentrate (rate ca 1 mL/min) to 4 mL in 60°C water bath. Remove KD from bath and let drain for 15 min. Remove concentrator tube and further concentrate to 1.0 mL under gentle stream of nitrogen (ultra high purity). Inject 8 μL into GC/TEA system and quantitate any peak with retention time corresponding to that of NDMA external standard. Calculate NDMA concentration on basis of peak heights.

For those samples containing more than 5 ppb NDMA, a larger sample is prepared as follows: Weigh 300 g beer into 500 mL round-bottom flask containing 48 g barium hydroxide and boiling chips. Distill into 500 mL separatory funnel, collecting 290 mL. Add 100 mL 5 N NaOH, mix, and let stand overnight. Extract distillate with three 100 mL portions of DCM, shaking each 1 min. Pass pooled extracts through 30 g anhydrous Na_2SO_4 (held in 60 mL sintered glass funnel and prewetted with DCM) into 500 mL KD with 4 mL concentrator tube attached. Wash Na_2SO_4 with 25 mL DCM into KD. Concentrate to 1 mL as previously described. Stopper and retain for column chromatographic cleanup.

Add 10 mL 6 N HCl in small increments to 6 g Celite with vigorous stirring (mixture will appear light and fluffy). Pack mixture in chromatographic column on top of glass wool plug, compressing to ca 2 in. Place small glass wool plug on top of packing. Add 50 mL *n*-pentane to column and drain. Transfer 1 mL sample extract to column with 20 mL *n*-pentane in four 5 mL increments. Drain column. Wash column with 200 mL 5% DCM in *n*-pentane and discard. Elute NDMA from column with 50 mL DCM and collect in 250 mL KD with 4 mL concentrator tube attached. Concentrate to 1.0 mL as previously

Table 1. *N*-Nitrosodimethylamine (ppb) in beer

Type of beer		No. of samples	Range	Av.
Imported	Beer	17	0.4–3.6	1.9
	Ale	2	3.3–3.4	3.4
	Dark, strong	1	ND[a]	ND
	Malt liquor	2	1.0	1.0
Domestic	Beer	37	ND–7.7	3.3
	Malt liquor	5	0.9–6.4	3.5
Total		64	ND–7.7	2.8

[a] None detected.

described. Transfer to Chromoflex sample tube and carefully concentrate to 0.1 mL under gentle stream of nitrogen. Stopper and retain for GC/MS analysis.

Results and Discussion

A total of 55 samples of beer, ale, and malt liquors were purchased at local retail markets in the Washington, DC area and 9 beers were obtained from the western coast of the United States. The results of the analysis of these samples are shown in Table 1. Of the 64 samples analyzed by GC/TEA for NDMA, 42 were domestic and 22 were imported beers. NDMA was found in 39 domestic samples at levels ranging from 0.6 to 7.7 ppb with an average of 3.6 ppb. In 21 imported beers, NDMA ranged from 0.4 to 3.6 ppb with an average of 1.9 ppb. NDMA was not detectable by GC/TEA analysis in 1 imported and 3 domestic beer samples. The detection limit of the GC/TEA system for NDMA was 0.4 ppb, based on a signal-to-noise ratio of 5:1. Of the 64 beers surveyed, 5 contained levels of NDMA greater than 5 ppb and were subjected to GC/MS confirmation. Recovery data for NDMA in beer by the GC/TEA detection and quantitation method have been previously reported (10).

Preliminary studies conducted to establish the retention time of NDMA and limits of detection of the capillary GC/MS system indicated that approximately 100 ng NDMA was necessary to obtain a representative full mass spectrum. This level of nitrosamine standard was necessary because of the limited storage capability of the data system used. Extraction of a beer sample larger than 25 g was thus required for the GC/MS confirmation to obtain a final concentration that would provide 100 ng NDMA in a 2 or 3 μL injection.

The initial attempt to confirm NDMA by GC/MS involved distillation of an alkaline solution of 300 g beer, extraction of the distillate with methylene chloride, and elution of the extract from an acid Celite column. This cleaned-up beer extract was then concentrated to 300 μL, and several microliters were withdrawn and analyzed by capillary GC/MS to determine the efficiency of the cleanup procedure near the retention time of NDMA. Examination of the total ion trace from this injection showed no interferences at exactly the same retention time as NDMA; however, a major component eluting just before this time was detected. Based on GC/TEA quantitation, a 10-fold concentration of the 300 μL extract would be required to bring the nitrosamine concentration within the detection range of the GC/MS system. At this concentration the level of the component eluting before NDMA would preclude the confirmation of the nitrosamine. Identification and removal of this interference was therefore essential to the confirmatory method.

Examination of the mass spectrum of the interference showed a high mass ion at m/z 88 and 2 fragment ions at m/z 45 (base peak) and m/z 43. Characterization of this compound as 3-hydroxy-2-butanone from the mass spectrum was later verified by comparing its full mass spectrum with that of a standard. The method was then modified to remove this compound by reacting the alkaline beer distillate overnight with 100 mL 5N NaOH as previously described (10), followed by extraction with methylene chloride and elu-

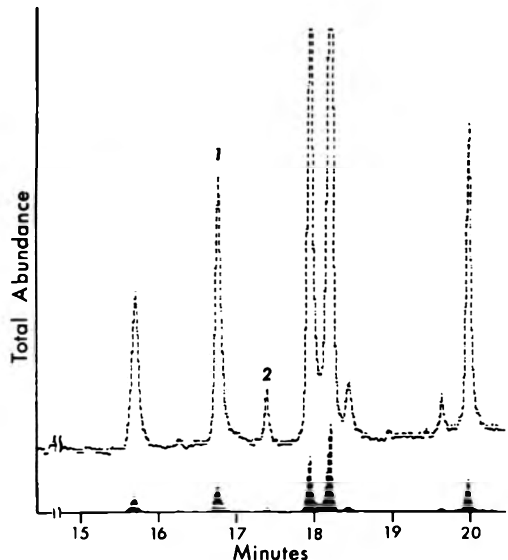

Figure 1. GC/MS total ion chromatogram of beer sample after analytical cleanup: 1, residual 3-hydroxy-2-butanone; 2, response for NDMA.

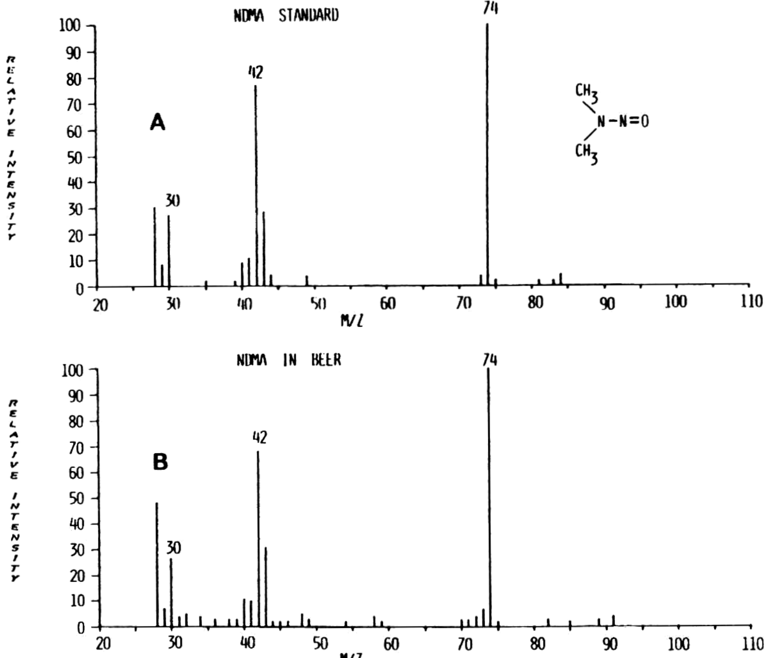

Figure 2. Mass spectra of (A) NDMA standard and (B) NDMA from beer sample.

tion from an acid Celite column as previously described. Subsequent studies have shown that significant amounts of 3-hydroxy-2-butanone can be removed by washing the beer extract 3 times with a 20% sodium bisulfite solution instead of carrying out the overnight sodium hydroxide reaction.

Figure 1 shows a total ion trace from a 2 μL injection of a typical 300 g beer extract cleaned up by this procedure. Component 1 represents the residual amount of 3-hydroxy-2-butanone remaining and component 2 is the response observed for NDMA. The final sample volume was 30 μL and the chromatogram represents the response of approximately 100 ng NDMA.

Figure 2 shows the full mass spectrum acquired from the injection of 100 ng NDMA standard and the spectrum of the nitrosamine obtained from the injection of 2 μL of a cleaned-up beer sample. The nitrosamine spectrum obtained from the beer sample exhibited all the ions observed in the spectrum of the standard. No additional ions of significant relative abundance were detected in the spectrum of the extract, indicating that there was no interference from compounds having the same or similar retention time. Comparison of the 2 spectra showed good agreement between the relative abundance of the ions in the sample spectrum and those obtained in the spectrum of the standard. Therefore, the identity of NDMA was confirmed, based on these characteristics and the agreement in the relative retention time of the standard and the sample.

In this report we have applied a capillary GC/MS technique, using full mass scans, to confirmation of the identity of NDMA in beer. Recent evidence suggests that NDMA is formed in malt during its manufacture when it is dried by air heated by direct flame. Alteration of the direct drying process has resulted in significant reductions in the quantity of NDMA in malt and, consequently, in beer. Our studies are being extended to include other foods dried in a manner similar to malt.

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Gravimetric Determination of Ethoxylated Mono- and Diglycerides in Bread

JAMES F. LAWRENCE, JAGANNATH R. IYENGAR, and HARRY B. S. CONACHER
Health and Welfare Canada, Health Protection Branch, Food Directorate, Food Research Division, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2

A gravimetric method is described for the quantitative determination of ethoxylated mono- and diglycerides (EMGs) in bread at levels as low 0.42% (dry sample). The air-dried pulverized samples are Soxhlet-extracted 22 h with an azeotropic mixture of n-propanol-water. The contents are evaporated to dryness, and then treated with 5% HCl in methanol to trans-esterify the fatty acids present which are then removed by petroleum ether extraction. The EMGs are subsequently precipitated from aqueous solution with phosphomolybdic acid in the presence of barium ions. The precipitate is weighed and compared with known amounts of standard carried through the same procedure.

Ethoxylated mono- and diglycerides (EMGs) are non-ionic surface-active ingredients permitted under the food additive regulations in the United States for use as dough conditioners (emulsifiers) in yeast-leavened bakery products, including bread, up to a level of 0.5% in the dry baking formula (1). There are no permitted food uses of EMGs in Canada (2); thus, methodology is required to monitor their presence in food products, particularly bread. A number of methods are available for the determination of non-ionic emulsifiers, including polyoxyethylene esters (3–6) and polyoxyethylene glycol (7). Methodology for these and other classes of emulsifiers has been reviewed (8–10). The phosphomolybdic acid barium complexation reaction was originally used by Shaffer and Critchfield (7) for the colorimetric determination of polyethylene glycols, and was later used in a gravimetric procedure (11). Since then, this approach has been applied with modifications to polysorbate 60 (12, 13) and polysorbate 80 (14, 15). It is also the basis of the method adopted by the U.S. Food and Drug Administration for EMG.

In our experience, the method used by the U.S. Food and Drug Administration (1) has not been entirely satisfactory, mainly because the technique is tedious and time-consuming, requiring a number of extractions, transfers, and evaporations. This report describes a gravimetric method based on phosphomolybdic acid barium precipitation (14) that makes use of a very different extraction procedure in which the analyst's actual working time is significantly reduced and only 3 transfer steps are required from beginning to end.

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Experimental

Reagents

(a) *Solvents.*—Glass-distilled.

(b) *Hydrochloric acid.*—Gaseous.

(c) *Barium chloride, dihydrate.*—10% aqueous solution.

(d) *Phosphomolybdic acid.*—10% aqueous solution.

(e) *Trans-esterification solution.*—5% HCl in methanol. Pass HCl gas through methanol previously dried over Na_2SO_4 (anhydrous), until 5% weight increase occurs.

(f) *Ethoxylated mono- and diglyceride (EMG) standards.*—Dissolve 5 g standard in 50 mL methanol. Dilute 10 mL aliquot 10-fold for spiking purposes (i.e., 1 mL solution = 10 mg EMG) with methanol.

Sample Analysis

Weigh 10 g dried, freshly ground bread in Soxhlet thimble. Extract 22 h with azeotropic mixture of 1-propanol-water (72 + 28). Remove round-bottom flask and evaporate solution to thorough dryness with a rotary flash evaporator at 60°C. Residual water may be removed azeotropically by adding ca 100 mL methanol and repeating evaporation. Extract residue in the flask with four 20 mL portions of $CHCl_3$ by gently swirling and decanting into clean 250 mL round-bottom flask. Evaporate solution to dryness with flash evaporator and add 25 mL trans-esterification solution. Reflux 1 h; then evaporate to dryness. Extract residue with three 20 mL portions of petroleum ether and discard ether. Dissolve remaining residue in ca 50 mL water, transfer to 400 mL beaker, dilute to 250 mL, and heat to boiling. Add, in order, 2 mL 3N HCl, 4 mL 10% barium chloride solution, and 4 mL 10% phosphomolybdic solution. Let stand overnight, covered with watch glass. Filter so-

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Table 1. Recovery of EMG from baked bread

Spiking level (dry sample), %	Moisture in baked sample, %	Wt ppt expected (10 g subs), mg	Wt ppt found (dupl.), mg	Recovery, %
0	36	0	0	0
0.42	35	208	100, 104	48.1, 50.0
0.77	32	389	200, 201	51.4, 51.7
1.12	33	560	254, 249	45.4, 44.5

lution through preweighed Gooch crucible. Rinse beaker with 75 mL water and pass rinse through crucible. Wash precipitate with 5 mL petroleum ether and dry crucible and contents 1 h in 105°C oven. Cool and weigh. Calculate weight of precipitate.

Determination of Calibration Factor

Pipet 3 mL EMG spiking solution (equivalent to 30 mg EMG) into 250 mL round-bottom flask. Evaporate on rotary flash evaporator to dryness. Add 25 mL trans-esterification solution and continue procedure, beginning with refluxing described above. Calibration factor, F, is calculated according to F = mg precipitate/mg EMG.

Calculation of Percent EMG in Sample

Calculate percent EMG in bread sample, using following equation:

% EMG = (mg precipitate/F) × (100/10 000)

Results and Discussion

Table 1 shows recoveries obtained on bread samples spiked *before baking* with 0.42–1.12% EMG. Although only about 50% of the EMG was recovered with 22 h Soxhlet extraction, it was considered satisfactory for our purposes because the values were reproducible. If higher recoveries are desired (or required), then Soxhlet extraction for 48 h (Smullin et al. (13)) with the same azeotropic mixture may be used. The extended extraction yielded, for example, 89% recovery of polysorbate 60 (13). We found 22 h to be a convenient alternative for EMG.

Spiking the bread samples after cooking was not realistic because recoveries approaching 100% were obtained even when Soxhlet extraction was carried out for only 4 h with a much less polar solvent mixture such as methanol–chloroform (1 + 2). This system, although satisfactory for other polyoxyethylene emulsifiers (3), was completely ineffective for samples spiked before the bread was cooked (recoveries, 4–15%). The difficulty with cooked samples appears to be the result of adsorption or complexation as a result of the cooking process. This was also observed for polysorbate 60 with cooked bakery products, unlike uncooked products such as salad dressing, shortening, or tomato paste where recoveries were essentially 100% (3, 13).

The trans-esterification reaction proved to be a simple technique for removal of free and esterified fatty acids. The method does not require saponification or numerous solvent partitions and transfers. Also, we found that the calibration factor was larger (average 5.2, range 5.0–5.3, 9 values) than that obtained after the saponification treatment (average 2.0, range 1.9–2.1, 12 values) over the range of 10–50 mg EMG. The reason for this difference is not known; however, both techniques yielded similar reproducibilities. It appears that a different complex stoichiometry results with the transesterification treatment. Elemental analysis of the precipitate would undoubtedly help in identifying its composition.

We consider the method described herein to be an attractive alternative to the saponification procedure mainly because it is simpler; it requires fewer sample manipulations and transfers and, thus, less time by the analyst. Although recoveries after 22 h were only about 50%, the quantity of precipitate recovered was actually more than that obtained through the saponification procedure. Because of the advantages and potential for routine analysis, this method should be evaluated collaboratively.

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NICOLE F. HARDIN
U.S. Food & Drug Administration
4298 Elysian Fields Ave
New Orleans, LA 70122
(504/589-2471)

HERSHEL MORRIS
Louisiana Dept of Agriculture
PO Box 16390-A, University Station
Baton Rouge, LA 70893
(504/388-2755)

ANALYTICAL MYCOLOGY OF FOODS AND DRUGS

Comparison of Yeast and Mold Counts by Spiral, Pour, and Streak Plate Methods

MARILYN R. ZIPKES, JAMES E. GILCHRIST,[1] and JAMES T. PEELER[1]
Food and Drug Administration, Division of Microbiology, 1141 Central Parkway, Cincinnati, OH 45202

Foods with naturally occurring yeast and mold were used in a comparative study of 4 plating techniques. Oats, green beans, and cheese were incubated for 3 and 5 days at 25°C. Although 3 days of incubation would be sufficient for yeast, 5 days were necessary for the mold count. The percent recovery of yeasts and molds by the spiral and streak methods ranged from 200 to 357% compared with the pour plating method described in the Bacteriological Analytical Manual (BAM). The spiral method had the highest overall recovery and the lowest replicate plating error.

Counts of yeasts and molds are based on standard pour plate methods (1–3). Although streak plates are used to identify molds, numerical results are obtained by counting pour plates. Most research on yeast and mold procedures has been directed toward selecting and improving the productivity of media (4–7). Current methods have shown that various foods, such as wheat flour, corn meal, brown rice, split peas, pecans, walnuts, soybeans, and dried beans (8), contain toxigenic mold species. Because of the potential health hazards of mold-contaminated food, methodology for recovery and identification of viable yeasts and molds must be improved. In reports (9–11) which compared surface and pour plate counts for bacteria, the latter 2 studies referenced presented a procedure for automated surface plate count for bacteria, which gave equivalent recovery and saved time as well as money. Later studies (12, 13) tested the variability of this spiral plate count (SPLC) procedure among several laboratories. Because this method is now an AOAC official method (2) for bacteria, its applicability to yeast and mold analysis was tested.

This study compared the SPLC procedure with the current pour plate method (3). Our preliminary tests showed that the percentage recovery of yeasts and molds by the SPLC method was higher than that for pour plating. A pure culture of *Staphylococcus aureus* was used to verify that the SPLC and pour plating methods gave the same concentrations.

Because streak plate procedures (14) are used in Europe to count yeasts and molds, a streak plate procedure was included in this study. A comparison with the SPLC showed that surface plating gave higher recoveries of yeasts and molds. The streak plating method for isolation and enumeration of *S. aureus* in food, sec. **46.075-46.076,** with modifications for agar and incubation, was adapted for enumeration of yeasts and molds. Five-day counts were made on plates that had been counted at 3 days to determine whether moving the plates would cause mold spores to spread and result in higher counts. It was observed that pouring the agar immediately after pipeting the diluted sample onto a petri dish gave better recovery and less duplicate plate variance. This modified pouring procedure was also incorporated into the tests of methods for yeast and mold enumeration.

METHOD

Apparatus

(**a**) *Spiral plater.*—For use with 150 × 15 mm petri dishes and adjusted to deliver total volume of 0.035 mL/plate, secs **46.110-46.111** (Spiral Systems Marketing, Bethesda, MD 20014).

(**b**) *Transparent viewing grid.*—For use in counting plates and calibrating spiral plater, secs **46.113** and **46.116.**

Media

(**a**) *Antibiotic stock solution.*—Dissolve 500 mg chlortetracycline HCl in 50 mL sterile water. Antibiotic No 100235 (ICN Pharmaceuticals, Inc., Irvine, CA 92715) is satisfactory. Filter solution with 0.45 μm plain membrane disposable filter unit. Filter No. 245-0045 (Nalge Sybron Corp., Rochester, NY 14602) is satisfactory.

(**b**) *Agar.*—Potato dextrose agar (PDA, Difco) is satisfactory. Suspend 200 g potato infusion, 20 g dextrose, and 15 g agar in 1 L water. Heat

[1] 1090 Tusculum Ave, Cincinnati, OH 45226.
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Table 1. Geometric means of yeast and mold counts using various methods and incubation times

		Method			
Food	Incubation time (days)	BAM	Modified pour	Streak	SPLC
Oats	3	13 000	10 000	37 000	46 000
	3/5[a]	13 000	15 000	42 000	55 000
	5[b]	15 000	13 000	50 000	51 000
Green beans	3	250 000	170 000	500 000	620 000
	5[a]	240 000	220 000	580 000	350 000
	5[b]	310 000	190 000	530 000	650 000
Cheese	3	420 000	430 000	1 100 000	1 300 000
	5[a]	400 000	390 000	1 100 000	1 300 000
	5[b]	400 000	440 000	1 000 000	1 200 000

[a] Plates counted at 3 days, reincubated, and recounted at 5 days.
[b] Counts on plates incubated undisturbed for 5 days.

and boil until all ingredients are dissolved. Dispense in 100 mL portions and autoclave 15 min at 121°C. Final pH, 5.6 ± 0.2.

For surface plates, temper sterile agar to 45 ± 1°C, add 4 mL antibiotic stock solution/L PDA, and dispense by a Brewer pipet. Dispense 50 mL (SPLC) or 15 mL (streak) antibiotic-amended PDA into appropriate size petri dishes (150 × 15 mm SPLC, 100 × 15 mm streak).

For pour plates, dispense boiled PDA in 100 mL portions in dilution bottles, sterilize, and store. Remelt bottled PDA, temper to 45 ± 1°C, and add 0.4 mL antibiotic stock solution to each bottle. Pour 18–20 mL portions into 100 × 15 mm petri dishes after seeding petri dishes with sample dilutions. Do not heat medium after adding antibiotic.

Procedures

(**a**) *Spiral plate method.*—Secs **46.115-46.116.** Incubation time, temperature, and media differ from AOAC method for bacteria.

(**b**) *Streak plate method.*—Use glass rods, sec. **46.075.** At each dilution plated, aseptically transfer 0.1 mL of dilution to each PDA-amended agar plate. Spread inoculum over surface of agar, using sterile, bent glass streaking rods. Allow plates to dry ca 1 h. Invert and incubate (2).

(c) *Pour plate method.*—Secs **46.014-46.015.**

(**d**) *Modified pour plate method.*—Same as pour plate method (c), except pour agar immediately after pipeting each dilution into petri dishes.

The yeast or mold count was based on identification by morphology; in each product observation was checked by Gram staining. The comparative counts of samples were used when 3 or 5 day count of standard pour procedure produced counts in 30–300 range. Standard counting rules for bacteria were used for all methods. Two dilutions of each sample were plated by SPLC method. Count of greater dilution was used unless count was <20 per plate as specified in SPLC counting rules.

Preparation of Samples

Weigh 50 g sample into sterile blender jar, add 450 mL dilution water, sec. **46.005(a),** and blend 2 min to obtain 10^{-1} dilution, sec. **46.114.**

Determination

Number and type of food samples analyzed were predetermined to statistically test a significant difference between methods and ability of each method to produce identical duplicate plates. For comparison, the 4 methods were run concurrently and 2 sets of duplicate plates per method were used. One set was incubated at 25°C, counted at 3 days, reincubated, and counted again at 5 days; one set was incubated undisturbed at 25°C and counted at 5 days.

Statistical Analysis

An analysis of variance (15) was performed on the $\log_{10}$ count. This transformation was used to normalize distribution of counts and to achieve homogeneity of variance. Means for the main effects (methods and incubation times) were analyzed by Duncan's test (16) to determine which means differed significantly. All tests of hypothesis were performed at $\alpha = 0.05$ level.

Performance of methods was judged on basis of recovery of natural yeasts and molds from products and replicate variability observed.

Results

Table 1 shows the geometric mean yeast and mold count/g for whole oats, green beans, and

Table 2. Summary of *F* tests from the analysis of variance [a]

Source of variation	Oats			Green beans			Cheese	
	Yeasts + molds	Yeasts	Molds	Yeasts + molds	Yeasts	Molds	Yeasts + molds	Yeasts
A Samples	20.05	23.18	26.51	204.03	238.16	6.22	872.24	707.24
B Incubation times	5.05	0.55*	28.40	1.26*	0.32*	13.67	0.31*	1.80*
C Methods	176.30	149.00	56.29	33.66	79.67	193.20	421.08	492.28
Interactions								
AB	3.11	3.65	3.09	2.33	2.09	2.65	0.56*	2.13
AC	3.57	4.52	3.37	1.73*	3.55	6.77	28.15	29.09
BC	1.05*	0.28*	0.57*	2.16	0.60*	2.03*	0.37*	1.28*
ABC	2.62	2.02	1.50	1.35*	2.38	1.65	0.91*	1.79*

[a] *F* ratio of all values significant at the $\alpha = 0.05$ level except those indicated by asterisks.

cheese, for 7, 7, and 9 samples, respectively. Counts of one set were made at 3 days and again at 5 days after reincubation; one set was counted only at 5 days of incubation. Pour plate samples with yeast and mold counts in the 30–300 range were used as the reference method for statistical analysis. Means ranged from 10 000 to 1 300 000 yeast and mold/g.

The analyses of variance results for yeasts, molds, and yeasts and molds were computed. Tests of hypotheses were made to determine if the means were equal for samples, incubation times, and method. For example, the hypothesis was tested ($\alpha = 0.05$) that the means of methods were equal. The interactions were also tested. A significant interaction between samples and methods showed that the differences among method means were of a different magnitude for some samples. *F* ratios for each test and significant results are noted in Table 2.

Methods for all groups differed significantly. Differences between incubation times were observed for mold counts in oats and green beans. Only one cheese sample produced a mold count. Yeast recovery did not differ over the 3 to 5 day incubation period. Yeast and mold distribution differed among samples, and 19 of 24 interactions involving samples were significant at the $\alpha = 0.05$ level (Table 2). Only the method–incubation time interaction for yeast and mold count of green beans was above the significance level.

Geometric means computed for the 8 groups and 4 methods are presented as a percent of the BAM (3) procedure (Table 3), and results that were significantly different ($\alpha = 0.05$) from the BAM procedure are noted. The streak count was significantly higher than the BAM pour procedure in all cases. The SPLC was significantly higher than the BAM method in 7 of 8 tests. The modified pour procedure was equal to or significantly less effective than the standard pour method in 7 of 8 tests.

The percent recovery of the 3 incubation conditions compared with the 3 day counts are presented in Table 4. Mold counts were significantly higher after 5 days than after 3 days.

Table 3. Yeast and mold recovery expressed as percent [a] of the BAM pour procedure [b]

Product	Test	Method [c]		
		Modified pour	Streak	SPLC
Oats	Yeasts + molds	93*	307	357
	Yeasts	86*	365	540
	Molds	89*	214	186
Green beans	Yeasts + molds	73	208	200
	Yeasts	62	280	243
	Molds	87	120	67
Cheese	Yeasts + molds	108*	275	325
	Yeasts	110	289	474

[a] Percents computed as 100 × (method geometric mean/geometric mean pour/g).
[b] BAM procedure = 100%.
[c] All values significant from the BAM pour geometric mean/g at the $\alpha = 0.05$ level except those indicated by asterisks.

Table 4. Yeast and mold counts expressed as a percent of 3-day incubation geometric means/g [a]

Product	Test	Incubation time (days) 3/5[b]	5[c]
Oats	Yeasts + molds	118[d]	123[d]
	Yeasts	108	108
	Molds	157[d]	157[d]
Green beans	Yeasts + molds	94	112
	Yeasts	106	112
	Molds	143[d]	150[d]
Cheese	Yeasts + molds	100	99
	Yeasts	101	107

[a] Counts at 3 days = 100%.
[b] Plates counted at 3 days, reincubated, and recounted at 5 days.
[c] Counts on plates incubated undisturbed 5 days.
[d] Significant from the BAM 3-day geometric mean/g at the $\alpha = 0.05$ level.

There was no significant change in yeast counts between 3 and 5 day incubation data.

Both surface plate procedures were superior to the pour plate in recovery and in replicate variation. Table 5 shows the replicate error for the 4 procedures and 3 products. The SPLC method had the lowest variance expressed in $\log_{10}$ counts. Overall estimates of variance for the pour, modified pour, and streak procedures were 4.5, 6.1, and 2.8, respectively, times the SPLC estimate; some of the variation, however, was due to spreading mold colonies. Variations of 55% of oat counts and 35% of green bean counts were due to molds. Duplicate plates were uncountable because of spreaders in 3.4, 5.3, 2.4, and 4.8% of the values for pour, modified pour, streak, and SPLC, respectively.

Discussion

The comparison of 4 methods showed that both surface plate techniques gave higher counts than pour methods. Recoveries by streak plates were 2–3 times higher than by the BAM (3) procedure. Recovery values ranged from 2 to 3.7 times higher than the BAM (3) for the SPLC. Although a preliminary study indicated that the modified pour plate procedure would increase the recovery, presented data show the opposite.

The spreading characteristics of yeasts and particularly molds made the colonies of these microorganisms more difficult to count than bacterial colonies. The variances shown in Table 5 correspond to the amounts of mold found in the products. There were more molds and a larger variance in oats than in green beans. Cheese with no mold had the lowest variance. The variance values for SPLC were less than those of other methods in all cases (0.00795; 69 degrees of freedom). This lower variance was probably due to the mechanically controlled deposition of the inoculum, which resulted in a decreasing density of colonies from the center of the agar plate. Thus, on each plate the area counted was the lower density area with more widely separated colonies. Although spreaders were a problem, the incidence of uncountable plates did not differ for the samples and methods examined in this study.

The present study confirms previous work on incubation times. If molds are suspected in a sample, a 5 day count is desirable unless the

Table 5. Estimates of replicate yeast and mold error for 3 foods and 4 procedures

Food	Procedure: BAM pour	Modified pour	Streak	SPLC
Oats	0.02698 (21)[a]	0.02808 (21)	0.02949 (21)	0.00962 (21)
Green beans	0.07855 (21)	0.12004 (21)	0.02840 (21)	0.01118 (21)
Cheese	0.00975 (27)	0.00894 (27)	0.01269 (27)	0.00415 (27)
Pooled variance for procedures	0.03593 (69)	0.04858 (69)	0.02258 (69)	0.00795 (69)

[a] Numbers in parentheses = degrees of freedom.

plates are overgrown. Products that contain only yeast could be incubated for just 3 days.

The spiral plating method was the method of choice for recovery and enumeration of yeasts and molds. The recovery was 2-3.7 times that of the BAM (3) procedure and the variance was 22% that of the BAM (3). With the streak method, sample distribution on the plates was even, but yeast and mold growth was denser along the edges of the petri dishes, and plates were difficult to count. The spiral plater dispensed decreasing amounts of sample on the plates, visual identification was improved, and selection of colonies for further testing was easier.

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TECHNICAL COMMUNICATIONS

Fast Cleanup of Difficult Substrates for Determination of Fenitrothion and Some Derivatives

PATRICK HACHÉ, RENÉ MARQUETTE, GILLES VOLPÉ, and VICTORIN N. MALLET[1]
Université de Moncton, Chemistry Department, Moncton, New Brunswick, Canada E1A 3E9

A simple method is described for the fast recovery of fenitrothion, an organophosphorus insecticide, from soil, chicken liver, urine, clams, and pine needles. The substrate is homogenized with acetonitrile or methanol, diluted with water, and passed through a column containing Amberlite XAD-7. Fenitrothion is recovered quantitatively by eluting with 4 portions of 25 mL ethyl acetate. After evaporation, the compound is determined quantitatively by gas-liquid chromatography with a flame photometric detector. The procedure is also suitable for some derivatives of fenitrothion, namely, fenitrooxon and *S*-methylfenitrothion. As low as 0.05 ppm of the parent compound may be determined.

The chemical insecticide fenitrothion [*O,O*-dimethyl-*O*-(3-methyl-4-nitrophenol)phosphorothioate] has been used on an almost annual basis to control spruce budworm (*Choristoneura fumiferana* Clemens) epidemics in eastern Canada. As a result, the chemical has been detected in several environmental substrates such as soil, pine needles, and softshell clams (1). Toxicologists studying its degradation in rats have had to analyze urine and liver specimens (2).

The development of the flame photometric detector (FPD) has greatly simplified the task of analyzing phosphorus-containing pesticides by requiring less rigorous cleanup procedures prior to gas-liquid chromatography (GLC). Nevertheless, when difficult substrates are involved, cleanup still remains tedious and time-consuming. For instance, in one particular method (3) for the quantitative determination of fenitrothion in pine needles or shellfish, the substrate (about 10 g) is macerated with ethyl acetate and centrifuged, and the supernate is extracted with hexane in the presence of acetonitrile. The extract is then purified by passage through a column containing activated Florisil, and the eluate is evaporated to a suitable volume before injection into a gas-liquid chromatograph. In another procedure (4), the substrate is mixed with acetonitrile, followed successively by extraction with hexane and charcoal column cleanup. With each method, an average of 2 samples per day can normally be processed.

This paper describes a method for recovering fenitrothion and some degradation products from difficult substrates, using Amberlite XAD-7 resin. The proposed method considerably shortens the complexity of the cleanup step, and the time required for a complete analysis is less than 2 h.

METHOD

Reagents and Apparatus

(a) *Pesticide standards.*—Prepare standard solutions and dilutions in methylene chloride (pesticide grade): fenitrothion (Forest Protection Ltd, Fredericton, NB, Canada), fenitrooxon (Chemagro Ltd, Kansas City, MO), *S*-methylfenitrothion and aminofenitrothion (synthesized according to literature (5)).

(b) *Amberlite XAD resins.*—British Drug Houses, Toronto, Canada. Prepare columns according to literature (6).

(c) *Gas chromatograph.*—Perkin-Elmer Model 3920 equipped with flame ionization detector (FID) and flame photometric detector (FPD). Use glass column, 183 × 0.64 cm (id) containing 3.6% OV-101 + 5% OV-210. Instrument settings, for fenitrothion only: column, 210°C; injection port, 250°C; interface, 250°C; nitrogen carrier gas, 60 mL/min. For separating fenitrothion and derivatives, set column temperature at 190°C.

Extraction and Concentration

Macerate 100 g clams (obtained locally), using Polytron mixer (Brinkmann Instruments, Rexdale, Canada). Homogenize 10 g subsample with 150 mL acetonitrile in Waring Blendor 5 min, filter through Whatman No. 202 paper, and rinse with acetonitrile. Dilute filtrate to 2 L with distilled water and pass through XAD-7 column at 130 mL/min. Wash column with 1 L distilled

[1] Address correspondence to this author.
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Table 1. Recovery (%) of fenitrothion from difficult substrates by using acetonitrile and Amberlite XAD-7

Substrate[a]	Concn, ppm[b]	Recovery, %
Soil	0.5	99, 93, 100
Chicken liver	5.0	106, 101, 100, 112
Male human urine[c]	0.5	100, 98, 102, 100
Softshell clams	5.0	100, 102, 107
Pine needles	5.0	100, 100, 100, 102, 100, 102, 100

[a] Final concentrate was light gold; up to 5 peaks were detected with FID (pine needles), while only one extraneous peak was detected with FPD.
[b] Final volume was 50 mL.
[c] Initial volume was 100 mL, diluted to 2 L.

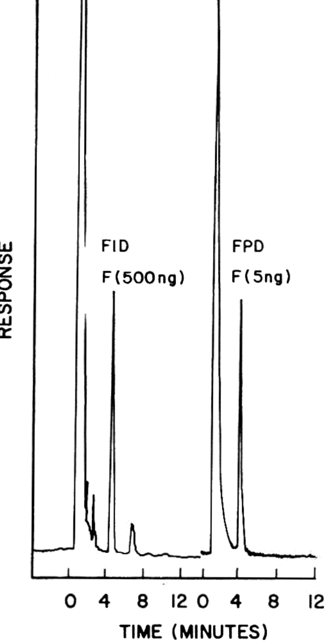

Figure 1. GLC chromatograms of fenitrothion in extract of pine needles with FID and FPD, respectively (F = fenitrothion).

water; then elute with four 25 mL portions of ethyl acetate at same flow rate. Concentrate eluate to desired volume (50 or 5 mL) for GLC.

Fortification Procedure

Fortify substrates with appropriate standards at desired concentration prior to homogenizing step.

Gas Chromatography and Quantitative Analysis

Inject 5 μL aliquots of appropriate standard solutions in GLC system and establish calibration curve. Inject 5 μL aliquot of final concentrated extract. Inject standard corresponding to approximate relative concentration. Calculate concentration directly from peak heights.

Results and Discussion

The intention in this study was to develop a fast and efficient procedure for the quantitative recovery of fenitrothion (and some derivatives) from difficult substrates, using Amberlite XAD resins. Previous studies (7) had revealed that fenitrothion could be recovered quantitatively from water by using an XAD-4 column. It was reasoned that if a difficult substrate could be transformed into a suitable "aqueous extract," then rapid column cleanup would be possible. This was achieved by diluting the original extract to 2 L with water to minimize the solvent effect on the solute percolating down the column.

The first step involved choosing a suitable extraction solvent, preferably one miscible with water. The following solvents were tested: acetone, acetonitrile, methanol, 2-propanol, water, ethyl acetate, ethanol. For comparison purposes, pine needles were used, the initial extract was diluted to 2 L with water, and the aqueous solution was passed through an XAD-4 column which was then eluted with three 30 mL portions of ethyl acetate. The initial extract was cleaner with acetonitrile or methanol (as determined by GLC with FID) and the former was used for all extractions at the 5 ppm level, but, as shall be discussed later, methanol was more suitable at very low concentrations.

The second step was to choose a proper resin. In previous studies (8) XAD-2, XAD-4, and XAD-7 had been promising. Amberlite XAD-7 was chosen because of better cleanup (as determined by FID) and increased recovery. Finally, a proper elution solvent had to be selected. Ethyl acetate had been used successfully before but other solvents, namely, toluene, chloroform, methylene chloride, and benzene were tested. Ethyl acetate was finally chosen because of reproducible recoveries, although good results were also obtained with methylene chloride or chloroform. The latter solvent gives more complete recoveries but its use was abandoned because of the potential health hazard.

Other experimental conditions were optimized. In general, reproducibility was better with a 12 cm XAD column, although a shorter column could be used with less difficult sub-

Table 2. Simultaneous recovery of fenitrothion and derivatives (%) from various substrates by using acetonitrile and Amberlite XAD-7

Substrate	Fenitrothion, 5 ppm	Fenitrooxon, 50 ppm	S-Methyl-fenitrothion, 50 ppm	Aminofenitrothion, 10 ppm
Softshell clams[a]	94	89	102	0
	107	83	102	0
Pine needles[a]	79	67	75	0
	83	70	84	0
	5 ppb	50 ppb	50 ppb	10 ppb
Water[b]	94	100	98	96
	93	97	95	94

[a] Final volume 50 mL.
[b] One liter passed directly through column.

strates such as urine and water. Initial dilution to 2 L was preferred because of improved recovery. Washing with 1 L water did improve cleanup of the final sample. Additional cleanup such as hexane and/or Florisil treatment (a Florisil column cleanup before the XAD column) was an improvement but at the expense of time.

Overall, more than 100 experiments were performed before the extraction procedure described herein was finally accepted. With this procedure, excellent recoveries were obtained for fenitrothion in soil, softshell clams, chicken liver, urine, and pine needles (Table 1). An idea of the reproducibility of the technique can also be obtained from the data in Table 1. For instance, with pine needles a standard deviation of only ±1.08 may be calculated.

The degree of cleanliness of the final extract can be better appreciated by looking at the GLC chromatograms presented in Figure 1. Using the FID, only a few peaks appear with a sample of pine needles. Similar results were obtained with the other substrates. With FPD, the chromatogram shows only the peak for fenitrothion (see Figure 1) without interference from co-extractives. The column temperature was set at 210°C to speed the separation.

The method is also acceptable for a mixture of fenitrothion and derivatives such as fenitrooxon and *S*-methylfenitrothion in pine needles, water, and softshell clams although recoveries for fenitrothion in pine needles were somewhat lower

Table 3. Recovery (%) of fenitrothion from difficult substrates by using methanol and Amberlite XAD-7

Substrate	Concn, ppm[a] 0.5	0.05
Soil	95	99
	100	95
	100	
Pine needles	96	88
	99	86
Male human urine	95	94
	103	90
Softshell clams	92	89
	98	92
Chicken liver	85	70
	88	75

[a] Final volume 5 mL.

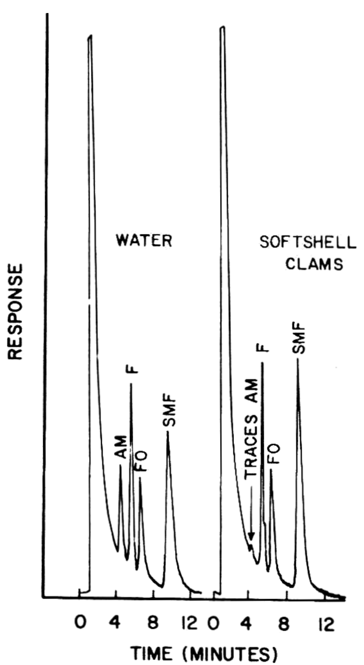

Figure 2. GLC chromatograms of fenitrothion and 3 derivatives extracted from water. AM = aminofenitrothion; F = fenitrothion; FO = fenitrooxon; SMF = *S*-methylfenitrothion.

than anticipated. Aminofenitrothion could not be extracted from either softshell clams or pine needles (Table 2) for reasons unknown. Typical GLC chromatograms showing fenitrothion and the 3 derivatives extracted from water and softshell clams are depicted in Figure 2. The column temperature was lowered at 190°C to improve resolution. The chromatograms demonstrate the absence of interfering co-extractives and good resolution of the peaks. The separation of fenitrothion by GLC, from known derivatives, has been reported in the literature (9).

Some of the experiments were initially carried out at higher concentrations (0.5 or 5.0 ppm) to establish optimum experimental conditions without fear of not detecting the solute. Further experiments at lower concentrations confirm that the method is acceptable (Table 3). Methanol instead of acetonitrile was used as extraction solvent because of better overall recoveries at the 0.05 ppm level.

The entire procedure from extraction of a substrate to injection into the chromatograph requires approximately $1\frac{1}{4}$ h, which is considered to be an important improvement in timesaving.

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Gas-Liquid Chromatographic-Thermal Energy Analyzer Method for *N*-Nitrosodiethanolamine in Cosmetics

DONALD B. BLACK, ROBERT C. LAWRENCE, EDWARD G. LOVERING, and JAMES R. WATSON[1]
Health and Welfare Canada, Bureau of Drug Research, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2

A gas-liquid chromatography-thermal energy analyzer (GLC-TEA) method has been developed for the quantitation of *N*-nitrosodiethanolamine (NDELA) in a variety of cosmetic products. Samples are cleaned up by a preliminary shakeout of an aqueous mixture of the cosmetic with ethylene dichloride, followed by removal of other polar and nonpolar constituents from the aqueous layer with commercial extraction columns, separation cartridges, and selected solvents. Common transnitrosating 1,3-diol preservatives sometimes found in cosmetics were eliminated after conversion to less polar cyclic boronates. The isolated NDELA fraction was trimethylsilylated at room temperature and the product was subjected to GLC-TEA, with *N,N'*-dinitrosopiperazine (DNPiz) used as the internal standard. Recoveries of NDELA from spiked samples usually ranged from 60 to 90%. The limit of detectability was about 5-10 ppb. The identity of NDELA was confirmed by GLC-mass spectrometry.

Since the discovery by Fan et al. (1) of the carcinogenic compound *N*-nitrosodiethanolamine (NDELA) (2) in some cosmetic products formulated with di- and/or triethanolamine, several investigations have been carried out to establish the nature and source of the nitrosating agent. Recent studies (3) have shown that the antimicrobial agent 2-bromo-2-nitro-1,3-propanediol (BNPD) can nitrosate both di- and triethanolamine in alkaline media, presumably by liberating nitrite ion which then attacks ethanolamine to form NDELA. Similar results and conclusions were reached by Ong and Rutherford (4). Fan et al. (1) examined a total of 31 cosmetics, hand and body lotions, and hair shampoos for NDELA and found levels which varied from 1 to 48 000 ppb, the latter in a facial cosmetic. Later studies conducted at the U.S. Food and Drug Administration (5) showed that, of 317 cosmetic products analyzed for NDELA contamination, 22 exhibited levels above 2000 ppb; the highest values were found for preparations that contained di- or triethanolamine or a derivative thereof and the preservative BNPD. These data coupled with the demonstrated absorption of NDELA across the skin and detection in the urine (6) indicate the need for a sensitive, specific, and robust method for the detection and quantitation of NDELA in a wide variety of cosmetic matrices.

This paper presents a gas-liquid chromatographic-thermal energy analyzer (GLC-TEA) method which involves a relatively rapid sample cleanup sequence, followed by elution of the NDELA as the trimethylsilyl derivative (NDELA-TMS), and quantitation to the low ppb level with reference to *N,N'*-dinitrosopiperazine (DNPiz) as internal standard. GLC-mass spectroscopy (MS) is used for NDELA identity confirmation as required.

METHOD

Apparatus

(**a**) *Gas-liquid chromatograph-thermal energy analyzer (GLC-TEA).*—Hewlett-Packard Model 5840 A recording gas chromatograph interfaced to Thermo Electron Corp. Model 502 thermal energy analyzer. GLC system was equipped with a 3.05 m (10 ft) × 4.0 mm id glass column packed with 3% OV-225 on 80-100 mesh Chromosorb WHP. Detector end of column was connected with a short length of nickel 200 tubing (1.6 mm od × 0.5 mm id, ca 10 cm) to inlet side of a stainless steel, gas-lined heated collection vent (Hewlett Packard option C-68). Temperature of collection vent was controlled by GLC keyboard through an auxiliary heating circuit card (Hewlett Packard part 18858-B). Exit port of vent assembly was interfaced to TEA system with 4 cm length of nickel 200 tubing (2.0 mm id) wrapped with heating tape and kept at temperature sufficient to maintain optimum peak shape and TEA response. TEA output signal was matched to input of data processor by means of a 10 V to 1 V resistive attenuator and an A/D converter unit (Hewlett Packard part 18871-A). TEA attenuation was 2048.

Operating conditions: argon carrier gas 20 mL/min; injector 240°C; collection vent 240°C;

[1] Address correspondence to this author.

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column programmed from 180°C for 6.5 min to 240°C at 7°/min, held for 2.5 min; TEA pyrolyzer 475°C; attenuation on data processor ×8. TEA stainless steel cold trap was immersed in a 1 L cryogenic stainless steel Dewar flask (Fisher Scientific Ltd) half-filled with liquid nitrogen (−195°C).

(b) *Rotary evaporator.*—Büchi Rotavapor-R (Fisher Scientific Co. Ltd, Montreal, Quebec, Canada).

(c) *Centrifuge.*—Clinical centrifuge, IEC Model 428, 3300 rpm maximum (Central Scientific Co. of Canada Ltd, Mississauga, Ontario, Canada).

Reagents

(a) *Methyl ethyl ketone.*—Distilled in glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI).

(b) *Ethylene dichloride.*—Distilled in glass (Burdick & Jackson Laboratories, Inc.).

(c) *Acetonitrile.*—Distilled in glass (Caledon Laboratories Ltd, Georgetown, Ontario, Canada).

(d) *Sodium-L-ascorbate.*—Sigma Chemical Co., St. Louis, Mo.

(e) *1-Butaneboronic acid.*—Aldrich Chemical Co., Montreal, Quebec, Canada.

(f) *Silylating agent.*—*N,O*-Bis(trimethylsilyl)-acetamide (BSA) (Chromatographic Specialties, Brockville, Ontario, Canada).

(g) *Disposable extraction columns.*—20 mL Clin Elut, manufactured by Analytichem International, Harbor City, CA (Fisher Scientific Co., Ltd, Ottawa, Ontario, Canada) or Preptube Cartridges, type 117 (Thermo Electron Corp., Waltham, MA).

(h) *Silica cartridges.*—Sep-Pak (Waters Scientific Ltd, Mississauga, Ontario, Canada).

Solutions

(a) *Stock nitrosamine solutions.*—NDELA in ethanol (100 μg/mL) and DNPiz in acetone (100 μg/mL) (Thermo Electron Corp.). These materials are reported to be carcinogenic.

(b) *Internal standard solution.*—DNPiz in acetonitrile (1.00 μg/mL) prepared by diluting stock solution. Keep in septum-sealed vial protected from light.

(c) *Spiking solution.*—NDELA in ethylene dichloride (1.00 μg/mL) prepared by diluting stock solution. Keep in septum-sealed vial protected from light.

(d) *Calibration solution.*—Dispense exactly 1.00 mL (1.00 μg NDELA) spiking solution by syringe into 50 mL round-bottom flask and remove solvent by rotary evaporation (bath at 40°C). Treat residue with 0.3 mL BSA and 1.00 mL internal standard solution. Let react at room temperature 15 min with frequent agitation. Prepare fresh daily and protect from light.

GLC-TEA System and Linearity Check

Using method described above for preparation of calibration solution, make 5 standard solutions, using exactly measured volumes of spiking solution equivalent to about 0.5, 1, 3, 5, and 7 μg NDELA. Inject 8 μL of each silylated solution in duplicate into GLC-TEA system and compute mean relative response factor, relative to internal standard, and its relative standard deviation. Latter value should not exceed ±2.5%. Under temperature program conditions stated, retention times for NDELA and internal standard should be ca 4.4 and 12.5 min, respectively. All peaks should be sharp and off-scale (except for NDELA in the 0.5 μg standard solution) and baseline should exhibit no significant rise during 17.6 min run.

Procedure

Accurately weigh ca 2 g cosmetic product and ca 50 mg sodium ascorbate into 25 mL screw-cap tube (125 × 20 mm). Pipet 3.0 mL water (deionized and residue-free) into tube. Add ca 1 g NaCl and 0.1 mL glacial acetic acid to aqueous mixture and shake vigorously by hand for 3 min with 15 mL ethylene dichloride. Centrifuge mixture 10 min. If upper aqueous layer is not clear or not well separated, add additional 0.2 mL acetic acid, shake, and recentrifuge.

With Pasteur pipet, carefully transfer entire upper aqueous layer to Clin Elut column and let soak in 5 min. Elute NDELA from column by passing five 20 mL portions of methyl ethyl ketone through column, waiting at least 4 min between additions. Slowly pass combined eluates through silica Sep-Pak cartridge, fitted to 50 mL Luer-Lok glass syringe, into round-bottom flask. Pass additional 10 mL methyl ethyl ketone through Sep-Pak into round-bottom flask.

Evaporate methyl ethyl ketone solution to ca 20 mL on rotary evaporator (water bath maintained at 40°C). Add ca 25 mg 1-butaneboronic acid, let mixture react 15 min at 40°C and evaporate solution to dryness on rotary evaporator. Dissolve residue in 50 mL ethylene dichloride with vigorous shaking and pass solution through fresh silica Sep-Pak cartridge; discard eluate. Elute trapped NDELA from cartridge with 15 mL anhydrous ethanol into 50 mL round-bottom flask and remove solvent on rotary evaporator at 40°C.

React residue in flask with 0.3 mL BSA and 1.00 mL internal standard solution at room temperature for 15 min. Inject 8 μL calibration solution and silylated sample solution into GLC-TEA system. Compute NDELA level in ng/g (ppb) in cosmetic with reference to calibration solution.

Recovery Efficiency Determination

Spike a second accurately weighed portion of cosmetic with amount (in ppb) of NDELA similar to that found initially. If no NDELA or only a trace (<15 ppb) is detected, spike with ca 0.25–0.50 μg (ca 125–250 ppb). Adjust total volume of ethylene dichloride in extraction tube to 15 mL. Analyze spike sample and calculate recovery efficiency, using following equation:

$$\text{Recovery efficiency, \%} = [(C_T - C_I) \times 100]/C_S$$

where C_T, C_I, and C_S = ppb NDELA total, initial, and spiked, respectively.

Results and Discussion

Preliminary cleanup of the cosmetic was carried out by extraction with ethylene dichloride. Under the present experimental conditions, this solvent removed most of the nonpolar components but extracted less than 5% of the NDELA present in the water. Sodium ascorbate was added to the mixture in the extraction tube to inhibit in situ nitrosation (1, 7, 8) and NaCl was used to prevent formation of emulsions (9). A small quantity of acetic acid enhanced the solubilization of NDELA into the aqueous layer (>95%) (9) and promoted a cleaner phase separation on centrifugation. The entire aqueous extract rather than a representative portion was transferred to the extraction column because cosmetics (particularly shampoos) are formulated with varying amounts of water and the exact volume of the total aqueous layer from a given sample is uncertain. The error associated with transfer of the entire aqueous layer was estimated to be less than 5%. In most cases, the separation of layers was clear-cut.

NDELA was isolated from water and most other polar cosmetic ingredients by passing methyl ethyl ketone through a commercial hydrophilic extraction column containing the aqueous sample extract and then through a silica separation cartridge. The elution of NDELA from the column with five 20 mL portions of methyl ethyl ketone was verified experimentally to afford recoveries of about 85% for amounts in the range 0.5–50 μg. A parallel study indicated that elution of NDELA from the Sep-Pak cartridge was close to 100% with 100 mL methyl ethyl ketone; other more polar materials were at least partially retained as indicated by the larger amount of residue when no cartridge was used. Remaining less polar compounds were removed on a second cartridge with 50 mL ethylene dichloride wash, leaving the NDELA trapped on the cartridge. To quantitatively remove the trapped NDELA a more polar solvent such as ethanol was required. Only 10 mL was needed to completely elute as much as 75 μg NDELA.

Many cosmetic preparations contain (usually at concentrations <0.1%) the preservative 2-bromo-2-nitro-1,3-propanediol (BNPD) which is pyrolyzed (underivatized or in the silyl form) in the TEA furnace at 475°C to give products whose signals could interfere with accurate quantitation of the NDELA peak. 1,3-Diols such as BNPD and propylene glycol which is also used as a cosmetic ingredient can be easily converted at room temperature in methyl ethyl ketone with 1-butaneboronic acid to less polar 6-membered cyclic boronate derivatives (10, 11) which can be separated on a silica cartridge from the highly polar NDELA. An investigation of mixtures of BNPD (10–15 mg) and NDELA (0.5–50 μg) showed that 100 mL ethylene dichloride eluted virtually all BNPD boronate but more than 96% NDELA was retained. Another antibacterial agent, 5-bromo-5-nitro-1,3-dioxane (BNDX), a cyclic derivative of BNPD, exhibited elution characteristics similar to those of BNPD boronate.

Solutions for injection were prepared by rotary evaporation of the solvent (ethylene dichloride, methyl ethyl ketone, ethanol) followed by treatment of the residues with BSA and internal standard solution. Comparison of several NDELA solutions prepared in this manner with solutions of the same concentration made by direct dilution of NDELA and internal standard stock solutions with acetonitrile before the silylation step demonstrated that no loss of NDELA occurred on rotary evaporation of the solvent when the heating bath was maintained at 40°C.

While it has been reported (6) that NDELA can be chromatographed without prior derivatization on an OV-225 column, sensitivity and peak shape were improved when the hydroxyl groups were silylated (12) to give the corresponding ether (NDELA-TMS). BSA effected this conversion rapidly at room temperature. To preclude the possibility of adsorption or breakdown of NDELA-TMS on the surface of metallic carrier gas lines, nickel tubing which is claimed to

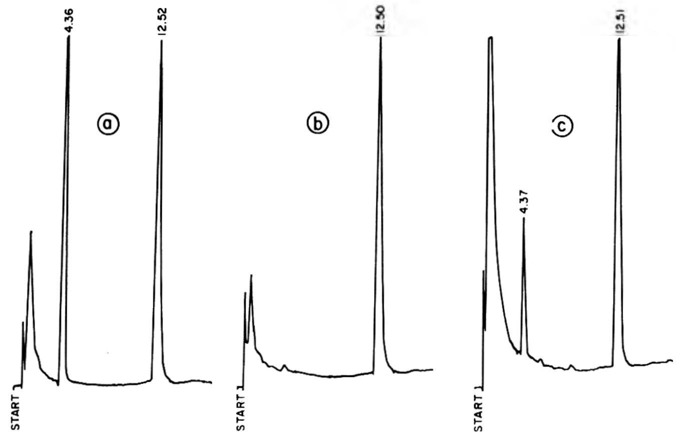

Figure 1. GLC-TEA chromatograms showing NDELA-TMS (retention time: 4.4 min) and internal standard (retention time: 12.5 min). a, calibration solution; b, cosmetic extract, no NDELA detected; c, same cosmetic spiked with 245 ppb NDELA, 159 ppb recovered.

combine the strength of stainless steel with the inertness of glass was used to interface the column to the TEA system. Since slush baths are cumbersome to prepare and are subject to fairly rapid changes in composition and temperature over a period of hours, the TEA cold trap was immersed in liquid nitrogen (−195°C), which was more convenient to handle. There was no appreciable difference in peak size when the trap was maintained at this temperature or at isopentane–liquid nitrogen or ethanol–liquid nitrogen slush temperatures (−160°C and −130°C, respectively). The moderate flow of heated argon gas through the trap was, in this work, sufficient to prevent the "freezing out" of the nitrosyl radical, although in view of this contingency, constant monitoring for a sudden decrease in NDELA response was advisable.

A system linearity study of several silylated solutions ranging in NDELA content from 0.5 to 7.0 μg/1.3 mL gave a mean relative response factor (NDELA to DNPiz: area ratio/weight ratio) of 0.491 with an RSD of ±1.7%. The response value which varied only slightly from day to day was about what was expected theoretically, the NDELA/DNPiz ratio of nitroso groups for equal weights being approximately 1 to 1.9. The minimum detectability limit was about 5–10 ppb (≃60–120 pg injected) calculated as NDELA but eluted as NDELA-TMS.

Sample chromatograms of the calibration solution, an unspiked sample with a non-detectable NDELA level, and the same cosmetic spiked with 245 ppb (159 ppb recovered) are presented in Figure 1. Generally, no difficulty was encountered in obtaining a clear aqueous layer during the initial extraction of the cosmetic in the screw-cap tube, although, in a few cases, particulate matter tended to gather at the phase interface. Nevertheless, almost all (>96%) of the water layer was usually available for transfer to the Clin Elut column. The column adsorbed the water, dyes, and most other polar materials to yield a colorless methyl ethyl ketone eluate when a waiting period of about 4 min between each solvent addition was observed. When the ethanol solution was evaporated before silylation, little or no resdiue was noted for creams or lotions, but a soapy material was present with shampoo preparations, perhaps due to sodium lauryl sulfate.

Because cosmetics contain a multiplicity of ingredients which differ widely in their physical and chemical properties, the partition of NDELA into the aqueous phase will vary (1) depending on the polarity and solubility of these ingredients. It was therefore important to determine the recovery efficiency for each cosmetic product studied. The spiking solution of NDELA was prepared in ethylene dichloride to simulate more closely the practical situation in that water must extract the nitrosamine from an immiscible medium or matrix The spiking solution was dispensed directly on the cosmetic, and shaken well before adding the other ingredients for the shakeout step. Recovery efficiencies were determined on 150 cosmetic products (creams, lotions, makeups, shampoos) by calculating the fraction (%) of spiked amount recovered. Of these recovery efficiency values, 123 were in the

Table 1. Recovery precision for cosmetic sample spiked with NDELA [a]

NDELA added, ppb	NDELA recd, ppb	Rec., %	NDELA added, ppb	NDELA recd, ppb	Rec., %
237	196	82.7	1064	826	77.6
242	177	73.1	1047	843	80.5
244	189	76.8	1010	946	93.7
243	189	77.8	1047	863	82.4
		Mean 77.6			Mean 83.5
		RSD ±5.1			RSD ±8.4

[a] No NDELA was detected in the unfortified cosmetic cream sample used in this study.

60–90% range, 15 varied between 40 and 60%, 8 were in the 90–110% range, with a few outliers ranging between 35 and 40%. Table 1 recoveries were determined for a cosmetic (no NDELA detected in unfortified sample) spiked at levels of 250 and 1000 ppb. Four determinations were performed at each level. RSD values for the 2 levels were 5.1% and 8.4%, respectively.

Four samples as well as the stock NDELA solution were subjected after silylation to mass spectral analysis on a Hewlett Packard GLC-MS system 5985 with ionization potential of 70 eV to confirm the identity of NDELA. The sample was cleaned up as described above except that trimethylsilylation was conducted with Tri-Sil concentrate in acetonitrile according to the method of Midha et al. (13). No internal standard was required. The column used for this work was a 1.2 m (4 ft) × 2 mm id glass column packed with 5% OV-101 on Chromosorb WHP operated isothermally at 140°C. In all cases, the fragmentation patterns were identical to those given in published spectra (14, 15) for the silylated derivative of NDELA.

Acknowledgments

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FOR YOUR INFORMATION

Rita A. Manning and her sponsor, R. F. Williams.

1981 AOAC Scholarship Awarded to Rita Manning of San Antonio, TX

Rita A. Manning, an outstanding chemistry student at the University of Texas at San Antonio, is the 1981 winner of a two-year, $1000 scholarship sponsored by AOAC.

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Meetings

April 13-15, 1982: 7th Annual AOAC Spring Training Workshop and Exposition, Fairmont Hotel, New Orleans, LA. Planned for this workshop are sessions on: drugs and antibiotics in feeds, food toxicology, forensic chemistry, pesticide residues, seafood quality, mycotoxins, environmental monitoring, fertilizers, sugars, laboratory automation, pesticide formulations, quality assurance, veterinary toxicology, hazardous waste monitoring, and analysis of toxicological substances. For additional information, contact co-chairmen: Nicole F. Hardin, U.S. Food and Drug Administration, 4298 Elysian Fields Ave., New Orleans, LA 70122; telephone 504/589-2471, or Hershel Morris, Louisiana Department of Agriculture, PO Box 16390-A, University Station, Baton Rouge, LA 70893; telephone 504/388-2755.

June 6-11, 1982: International Symposium on the Synthesis and Applications of

Isotopically Labeled Compounds, Hyatt-Regency Hotel, Kansas City, MO. The objective of the symposium is to provide a forum for the exchange of information between leading scientists involved in the synthesis and applications of isotopically (radio-active and stable) labeled compounds. The symposium will include a scientific exhibit. Topics will encompass synthesis, analysis, purification and storage of isotopically labeled compounds, and their applications in biomedical, clinical, and environmental studies, as well as metabolism, pharmacokinetics, and toxicology. For further information regarding submission of papers and registration, contact Alexander Susán, Scientific Secretary of the Symposium, c/o Midwest Research Institute, 425 Volker Blvd, Kansas City, MO 64110; telephone 816/753-7600, extension 268.

September 20–23, 1982: Symposium on Food Research and Data Analysis, Vokenasen Hotel, Oslo, Norway. Organized by the Norwegian Food Research Institute; sponsored by the International Union of Food Science and Technology, IUFoST. This symposium is intended to provide an interdisciplinary meeting ground for scientists interested in the development and use of computer-aided analysis of multivariate food research data. Agronomists, microbiologists, chemists, technologists, statisticians, marketing people, and psychologists with some experience in data analysis will be able to study the potential of more advanced analytical tools. Specialists in the fields of chemometrics, qualimetrics, and psychometrics will have an opportunity to "shake hands." The oral contributions will be given so that food scientists without advanced knowledge in mathematics or computer science can understand. Registration fee: N.Kr 1800 (≈US$360). Final registration: June 1, 1982. For more information, contact Symposium Secretariate: Norwegian Food Research Institute, Bjørn Eldstuen, PO Box 50, N-1432 Aas-NLH, Norway.

October 25–28, 1982: 96th Annual AOAC Meeting will be held at its new and larger "home", the Shoreham Hotel, Washington, DC. For more information, contact Kathleen Fominaya, AOAC, 1111 N 19th St, Arlington, VA 22209; telephone 703/522-3032.

Course Offered

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NEW PUBLICATIONS

Chemical Derivatization in Gas Chromatography. By J. Drozd. Published by Elsevier Scientific Publishing Co., PO Box 211, 1000 AE Amsterdam, The Netherlands; also available from Elsevier North-Holland, Inc., 52 Vanderbilt Ave, New York, NY 10017, 1981. 244 pp. Price US $58.50/Dfl. 120.00. ISBN 0-444-41917-9.

The five chapters comprising this book discuss reasons for using chemical derivatives in gas chromatography; some practical aspects of sample preparation and analysis of various derivatives; problems of identification and quantitative analysis; the most frequently used derivatives classified according to functional groups of the compound under analysis; and the derivatization and analysis of particular groups of compounds. The book should be of use to the novice and the proficient worker.

Impact of Toxicology on Food Processing. Edited by J. C. Ayres and J. C. Kirschman. Published by AVI Publishing Co., Inc., PO Box 831—250 Post Rd, East Westport, CT 06881, 1981. Approx. 500 pp. Prices approx.: U.S. & Can. $45.00, other countries $49.50. ISBN 0-87055-387-9.

An overview of toxicity in relation to food, food ingredients, additives, and processes is provided by this book which resulted from the Institute of Food Technology Basic Symposia Series. Major topics include the safety and regulatory aspects of additive toxicity, undesirable biological substances in foods, and toxins in relation to processing. Discussions examine colors, flavors, spices, bulking agents and fillers, vitamins, antioxidants, safety of irradiated foods, cyanogenic glycosides, aflatoxins and other mycotoxins, and antibiotics. This is a reference book for food technologists, chemists, microbiologists, and nutritionists involved in food safety, product development, and implementation of food regulations.

Prescott & Dunn's Industrial Microbiology, 4th Ed. Edited by G. Reed. Published by AVI Publishing Co, Inc., PO Box 831—250 Post Rd, East Westport, CT 06881, 1981. Approx. 1060 pp. Prices approx.: U.S. & Can. $59, other countries $65.00. ISBN 0-87055-374-7.

Because the field of industrial microbiology has grown immensely since the last edition of *Prescott and Dunn* 20 years ago, the 4th edition focuses on the industrial technology and applications involved in the microbiology of foods and food ingredients. Initial chapters outline fundamentals including microbial taxonomy, metabolism, and genetics; yeasts; and pure culture methods. The remaining chapters include coverage of microbial processes and functions in industrial production of cheeses, cabbage, cucumber, olive products, wine and brandy, beer, bakery foods, and distilled beverages. This is a guide and reference for food technologists, microbiologists, dairy chemists, cereal chemists, fermentation technologists, bioengineers, food production personnel, and those in research and development of food products and by-products.

Protein Quality in Humans: Assessment and in vitro Estimation. By C. E. Bodwell, J. S. Adkins, and D T. Hopkins. Published by AVI Publishing Co., Inc., PO Box 831—250 Post Rd, East Westport, CT 06881, 1981. Approx. 1060 pp. Prices approx.: U.S. & Can. $59, other countries $65. ISBN 0-87055-374-7.

This book describes the current status and potential applications of in vitro evaluation of nutritional quality of proteins for human consumption. The precision and accuracy available in estimates of protein nutritional value in human studies and through use of in vitro procedures is assessed. Topics discussed include importance of protein quality, assessment of protein nutritive value in humans, in vitro methods for assessing protein nutritional value, and evaluation of the validity and potential of in vitro assays for predicting the quality of proteins for human consumption.

Source Book of Flavors. By H. B. Heath. Published by AVI Publishing Co., Inc., PO Box 831—250 Post Rd, East Westport, CT 06881, 1981. Approx. 850 pp. Prices

approx.: U.S. & Can. $79.50, other countries $87.50.

Source Book of Flavors covers such areas as flavor chemistry, colorants, quality assurance, toxicology, and labeling. It includes data on natural flavoring materials, 325 plant materials, principal essential oils and organic chemicals used in flavorings, synthetic flavoring materials, aromatic chemicals, flavoring classified as GRAS by FEMA, and 350 flavoring formulations. The book is documented with over 3000 references.

Trends in Analytical Chemistry. Published by Elsevier Scientific Publishing Co., PO Box 211, Amsterdam, The Netherlands; also available from Elsevier North-Holland Inc., 52 Vanderbilt Ave, New York, NY 10017. Volume 1, 16 issues, March 1981 and monthly October 1981 to December 1982. Prices: personal edition US $42.50, £20.00(UK), 91.50 Dutch guilders (Europe) and 95.50 Dutch guilders (elsewhere); library edition (Volume 1 plus a compendium volume of archival material, hardbound, and indexed) US $133.25, 260 Dutch guilders worldwide.

Trends in Analytical Chemistry is a new international publication of short critical reviews, articles, and news on trends and developments in analytical chemistry.

CORRECTION

J. Assoc. Off. Anal. Chem. (1981) **64**, 311-318, "Paper Chromatographic Determination of Total Capsaicinoids in Capsicums and Their Oleoresins with Precision, Reproducibility, and Validation Through Correlation with Pungency in Scoville Units," by N. C. Rajpoot and V. S. Govindarajan, p. 315, right column, first line

Change ($P \leq 0.05$) to ($P > 0.05$)

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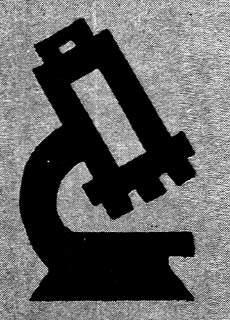

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